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# THE ESTABLISHMENT OF A CLINICAL CHEMISTRY QUALITY CONTROL PROGRAM*

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The purpose of this paper is to present the practical experiences of this laboratory in the establishment of a system of quality control in clinical chemistry. The essential part of the program is the daily determination of samples of known concentration for each type of analysis and the calculation of limits within which control values must fall before tests may be considered reliable or "in control."

The program has been designed for a chemistry department handling approximately 26,000 analyses per year performed by five full time, one part time, and one student technologist. First attempts at quality control were made about three years ago. At this time a simple means of processing and deep freezing plasma from out-dated Blood Bank bloods made available in quantity an inexpensive source of samples suitable as controls for some of the chemical analyses. Six months ago an all out effort was made to expand the program to provide controls for more types of analyses and to apply statistical methods to control values being obtained in order to "set limits" thus establishing true quality control.

Excellent help has been found in the account of a similar quality control system published by Freier and Rausch.[1] Statistical methods used were taken from a recent paper by Copeland.[2] The program at this time is incomplete as there are tests still not covered by controls due to difficulties in obtaining or preserving specimens. All problems concerning how to meet "out of control" situations have not been solved. However, it is hoped that the presentation of the program as it stands will be of value.

## QUALITY CONTROL PROGRAM

### Preparation and Use of Control Samples

Careful consideration has been given to the selection of solutions to be used as controls. No one solution has been found which will serve as control for all tests being performed. Factors such as availability, cost, keeping quality, and simplicity are important.

Throughout this paper these controls will be referred to as "control samples" in order to distinguish them from standard solutions used in

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the preparation of calibration curves. A list of the types of control samples and the tests for which each is used is given in the following table (Table I):

**TABLE I**

| Type of Control Sample | Test |
|---|---|
| 1. Pooled dialyzed plasma (Frozen) | Bilirubin, cholesterol, chloride, creatinine, serum total protein, albumin, globulin, uric acid, spinal fluid protein. |
| 2. Pooled serum (Frozen) | Alkaline phosphatase, phosphorus, sodium, potassium, urea. |
| 3. Protein free filtrate (Frozen) | Glucose (Somogyi-Nelson filtrates) Non protein nitrogen (Folin-Wu filtrates). |
| 4. Commercial preparations | Serum protein (Armour Bovine Albumin). |
| 5. Flocculation controls, positive and negative (Frozen) | Cephalin flocculation, thymol flocculation, thymol turbidity. |
| 6. Carbonate control (Sodium carbonate solution) | Carbon dioxide content. |

1. **Pooled Dialyzed Plasma** is prepared from out-dated Blood Bank bloods. Bloods to be used are carefully chosen, selecting only those showing no hemolysis and containing a minimum amount of chyle. The plasma is centrifuged to remove cells and clots. Since the blood bank plasma has been diluted approximately 20% by the addition of anticoagulants and preservatives, it is restored to normal concentration by dialysis. Chloride content is lost during the dialyzing process but is restored by adding a calculated amount of sodium chloride.

Dialysis is carried out in cellulose casing suspended in a 25% solution of polyvinylpyrrolidone (P.V.P.) in barbital buffer at pH 8.6. The barbital buffer is prepared by disolving 2.76 grams diethylbarbituric acid and 15.4 grams barbital sodium in one liter of distilled water. The cellulose casing, size 30/100, an inch and one half in width, is obtainable from the Visking Company, Chicago, Illinois. To concentrate two liters of plasma, three lengths of this casing, each 1½ to 2 yards long, are used. One end of the casing is securely fastened with a rubber band and the plasma is poured inside. The other end of the casing is similarly colsed and then it is laid out in a large vessel containing 1 to 1½ liters of the dialyzing agent. 5½ to 6 hours is usually required to concentrate two liters of plasma.

After dialysis is completed, the casings are quickly rinsed and dried and the contents rapidly drained into a flask. The plasma is then mixed and measured. At this point the total protein and cholesterol concentrations are determined. If the values are too low, further dialysis is carried out; if the values are too high, they are adjusted by a calculated dilution with distilled water. After this volume adjustment has been made, the chloride and uric acid concentrations are determined. Chloride in the form of sodium chloride is added to bring the value to approximately 100mE per liter. Uric acid content is increased by the addition of a calculated amount of a standard uric acid solution containing 1 mg. per ml.

The following table (Table II) shows typical values obtained during the processing of 2000 ml. of plasma. Dialysis time was 5½ hours.

**TABLE II**

| | Initial Conc. | Ideal Conc. | Conc. After Dialysis | Component Added | Final Conc. |
|---|---|---|---|---|---|
| Total Protein (g./100 ml.) | 5.1 | 7.5 | 7.3 | ..... | 7.4 |
| Albumin (g./100 ml.) | ... | ..... | 5.0 | ..... | 4.8 |
| Globulin (g./100 ml.) | ... | ..... | 2.3 | ..... | 2.6 |
| Cholesterol (mg./100 ml.) | 162 | 200—300 | 214 | ..... | 210 |
| Chloride (mE/liter) | 62 | 100 | 33 | 5.46g. NaCl | 98 |
| Uric Acid (mg./100 ml.) | ... | 4—6 | 1.2 | 3.mg. uric acid/100 ml. | 3.2 |

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After preparation the dialyzed plasma is divided into sample aliquots in small pyrex test tubes. These are stoppered and stored in a deep freeze unit. Each tube contains enough control sample for one day's work load. In practice one batch of prepared plasma has supplied this laboratory for about seven months. Control values over this period have been very consistent, showing the remarkable keeping quality of frozen plasma.

2. **Pooled Serum** is obtained by collecting and pooling all suitable sera left over from the daily work. Hemolyzed sera are not used. Sera from patients with slightly elevated alkaline phosphatase values are used as it is desirable for this test to set the value of the control sample in the range slightly above the normal value.

   Sera are placed daily in a large flask kept in the deep freeze. When a sufficient amount has accumulated, the content of the flask is thawed and centrifuged. The supernatant serum is then **thoroughly** mixed, divided into small aliquots and stored in the deep freeze.

3. **Protein Free Filtrate Controls** are collected in a similar manner by pooling Somogyi-Nelson or Folin-Wu filtrates daily and storing the pools in the deep freeze until sufficient amounts have accumulated. The importance of thoroughly mixing the large pool immediately before dividing it into small samples must be emphasized if consistent results are to be obtained.

4. **Commercial Preparations** useful as control samples are now readily available. Armour Bovine Albumin standard is used in this laboratory as a control sample for total serum protein determinations in addition to the frozen plasma control. This standard is kept at refrigerator temperature.

5. **Flocculation Controls** are prepared from a know positive and a known negative serum. Each serum is dispensed in 0.1 ml. and 0.2 ml. amounts in 100x15 mm. test tubes and stored in the deep freeze. For use as daily controls, one set of tubes is thawed and test reagents added directly. This system eliminates waste of serum and time.

6. **Carbonate Control** is a solution of sodium carbonate prepared in a concentration of 25 millimoles per liter. This solution is kept at room temperature.

### Daily Procedure For Use of Control Samples

Controls are run with each batch of determinations. Usually control samples are run in duplicate unless such a procedure is completely im-

practical. As most of the controls are preserved in the frozen state, specimens must be thawed and mixed completely before sampling to obtain consistent results. Although it sounds simple, the **thorough** mixing of samples must be emphasized. Studies in this laboratory have shown marked "layering effects" in serum or plasma that is allowed to thaw and sampled without mixing. For example, the protein value in the bottom layer will be about twice as high as that in the surface layer.

### Establishment of Limits

The use of a control sample with each analysis in the chemistry laboratory does not necessarily mean an effective quality control system unless definite rules are set as to the allowable variation in control values from day to day. This allowable variation will be referred to as "control limits." Such limits cannot be set without a thorough study of the reproducibility of the method in use under the **exact** working conditions of the laboratory.

To set limits on a control a statistical analysis of the results obtained on the sample for ten successive days is made. Whenever possible determinations are run in duplicate. The results over this ten day period will represent the work of several technologists who are also performing many other routine procedures. Although this data will show less precision than that of one technologist performing all the work on a single day, the calculated limits will represent the reproducibility of the method under the exact conditions of this laboratory.

Statistical studies of the data in this paper are based on the use of standard deviation as the unit of precision. Control limits are expressed as a function of the standard deviation.

When control samples are analyzed in duplicate, the standard deviation is calculated from the formula:

$$\text{Standard deviation} = \pm\sqrt{\frac{\text{Sum of the squares of differences between duplicates}}{2\ (\text{Number of Pairs})}}$$

When single determinations only are run on control samples, the standard deviation is calculated from the formula:

$$\text{Standard deviation} = \pm\sqrt{\frac{\text{Sum of the squares of differences from mean}}{\text{Number of measurements minus one}}}$$

Note: (See Appendum)

After the standard deviation for a control sample has been determined, the control limits to be demanded of future analyses are calculated according to an established statistical method[2] as the value of the mean plus or minus three times the standard deviation. For example, the mean value for a set of glucose determinations is 123 mg. per 100 ml. whole blood and the standard deviation calculated from a set of ten analyses in duplicate is 1.0 mg. per 100 ml. The control limits for glucose will

therefore be 123 mg. plus or minus 3 x 1.0 or 120 - 126 mg. per 100 ml. whole blood.

### Use of the Limits

A running record of the results of the quality controls is kept on a special sheet posted in a conspicuous place in the chemistry laboratory. The tests and the control limits for each are listed on this sheet with convenient spaces for entering daily results.

If any test fails to stay within limits, or is "out of control," the situation must be reported to a supervisor. The supervisor is then responsible for ferreting out the cause of the discrepancy. Ideally, in any "out of control," case all work should be repeated immediately. However, this is frequently a practical impossibility. At such times the supervisor may decide to postpone all reports until the following day. Another possibility is to repeat only the abnormal findings. If the cause of the trouble is a calculation error or an error in the analysis of the control sample itself, repeat work may be unnecessary.

For all methods, particularly new ones, the use of quality control limits is an excellent way of evaluating the reliability of a procedure. If a test consistently shows poor precision and accuracy, with more than an expected percentage of "out of control" runs, a replacement method must be sought.

### Other Control Procedures

In addition to the study and evaluation of control samples, several other features of the chemistry program in this laboratory are also part of the overall chemistry quality control system.

1. **Daily Calibration Curves:** Instruments used for colorimetric procedures are calibrated daily unless stable standards are not available. Calibration curves are prepared on the basis of at least two standards and sometimes even five for color reactions which do not adhere strictly to Beer's Law. Careful consideration is given to the selection and purchase of chemicals to be used as primary standards.
2. **Reference Methods:** An excellent means of quality control is the use of more than one method for determining the same component. However, this is usually too time consuming to be practical. This laboratory has found a simple, rapid, refractive index method described by Sundermann[3] useful for checking serum proteins determined by a micro-Kjeldahl method.[14]
3. **Calculation Checks:** Calculation mistakes rate high as a source of error. Ideally, all calculations should be checked by a second person. Practically this is difficult to accomplish. When time permits, this responsibility is assigned to the student technologist. For efficiency, routine calculation methods should be simplified as much as possible.
4. **Solution Checks:** Newly prepared solutions are checked against the solutions in use before the supply of the latter is exhausted. This is an important part of a good quality control system but a difficult one to maintain in a busy laboratory.
5. **Reporting and Repeating Abnormals:** All abnormal findings are reported daily to the pathologist in charge. "Confirmation Request" forms are available to all staff doctors. By use of these forms, the

laboratory will repeat free of charge any result which the physician in charge feels is not consistent with the clinical status of the patient.

6. **Distilled Water Check:** Distilled water is checked daily for impurities by means of a Barnstead Purity Meter. A test for chlorine in water using orthotolidine reagents is to be added in the near future.[4]
7. **Proficiency Surveys:** This laboratory maintains a subscription to a monthly proficiency survey conducted by Dr. F. William Sunderman, 1025 Walnut Street, Philadelphia, Pennsylvania.

## PROGRESS REPORT

The quality control program as outlined has been in effect for six months. Results of control samples have been evaluated twice and limits have been set according to the statistical method described. During the second evaluation, all results "off limits" according to the first evaluation were omitted. Results of the two studies are given in the following table (Table III).

**TABLE III**

| Test | Units | EVALUATION NO. 1 | | | EVALUATION NO. 2 | | |
|---|---|---|---|---|---|---|---|
| | | Mean | Standard Deviation | Limits (3 x S.D.) | Mean | Standard Deviation | Limits (3 x S.D.) |
| Alk. Phosphatase (5) | B.U. | 1.03 | 0.30 | 0.1—1.9 | 6.81* | 0.52 | 5.3—8.3 |
| Bilirubin (6) | mg/100 ml | 0.43 | 0.15 | 0.1—0.8 | 0.40 | 0.12 | 0.1—0.7 |
| Carbon Dioxide (7) | mM/l | 27.5 | 1.70 | 23—32 | 24.9 | 0.33 | 24—25 |
| Cholesterol (8) | mg/100 ml | 250 | 4.62 | 235—265 | 256 | 4.51 | 242—270 |
| Chloride (9) | mE/l | 98.2 | 0.73 | 95—101 | 96.5 | 0.50 | 95—98 |
| Creatinine (10) | mg/100 ml | .... | .... | ..... | 1.39 | 0.04 | 1.3—1.5 |
| Glucose (11) | mg/100 ml | 122 | 2.15 | 116—128 | 123 | 1.09 | 120—126 |
| Non-Protein N (12) | mg/100 ml | 41.0 | 1.70 | 36—46 | 39.3 | 1.71 | 34—44 |
| Phosphorus (13) | mg/100 ml | 5.63 | 0.15 | 5.1—6.1 | 3.93* | 0.14 | 3.6—4.2 |
| Potassium (14) | mE/l | .... | .... | ..... | 4.80 | 0.05 | 4.7—4.9 |
| Sodium (14) | mE/l | .... | .... | ..... | 144 | 0.87 | 141—147 |
| Total Protein (15) | g/100 ml | 7.35 | 0.31 | 6.5—8.2 | 7.28 | 0.22 | 6.7—7.8 |
| Albumin (15) | g/100 ml | 4.68 | 0.24 | 4.0—5.4 | 4.63 | 0.19 | 4.0—5.2 |
| Globulin (15) | g/100 ml | 2.75 | 0.25 | 2.0—3.5 | 2.66 | 0.34 | 1.7—3.7 |
| Total Protein (15) (Armour Standard) | g/100 ml | 6.04 | 0.13 | 5.6—6.4 | 6.00 | 0.08 | 5.7—6.3 |
| Uric Acid (16) | mg/100 ml | 3.34 | 0.42 | 2.1—4.4 | 3.34 | 0.44 | 2.0—4.6 |

* New Control Sample

From the data in Table III, valid statements can be made concerning the precision of methods in use. For example, glucose and chloride show good precision. Procedures showing less satisfactory reproducibility, are now being reviewed for technical difficulties and necessary changes in technique. Non-protein-nitrogens have been replaced by urea determinations. The marked improvement in the second $CO_2$ evaluation is due to the correction of an error in the introduction of the sodium carbonate standard, as used in the Van Slyke method.

Use of the limits has pointed out the fact that 19% of cholesterols have been "out of control." A new method has been worked up and will be started shortly.

Use of a calculation check by a second person has prevented errors at least six times since the start of the program. Faulty solutions have been picked up twice.

The benefit to be derived from a quality control system in clinical chemistry is clearly evident. However, it must be emphasized that to

outline the program is one thing; to maintain it is another. Chemistry laboratories must be adequately staffed by competent technologists, if procedures for calculation checks, solution checks, and preparation of accurate control samples are to be faithfully followed.

The problem is what to do in "out of control" situations is frequently a very difficult one to solve. In the so-called routine laboratory directly concerned with the diagnosis and treatment of patients a delay in results and the necessity for repeating large series of tests can create a very real problem for the laboratory and for the physician awaiting results. Since reliable results are essential, no quality control program is complete which does not plan for meeting such emergencies.

## SUMMARY

The establishment of a quality control program for a clinical chemistry laboratory is described.

Methods are presented for the preparation and use of control samples.

A statistical method is outlined for determining limits of accuracy.

Practical experience with and benefits to be derived from clinical chemistry quality control are reviewed.

## ACKNOWLEDGMENT

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## APPENDUM

Since the first presentation of this paper, continued studies of the quality control data from this laboratory have shown that the calculation of **all** standard deviations according to the formula for single determinations, and the omission of the formula for duplicates, is more satisfactory and practical for most clinical chemistry laboratories.

# NOTES ON THE GARROW TEST—A RAPID TEST FOR INCREASED COLD AGGLUTININS*

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The titration of cold agglutinins is a routine laboratory procedure frequently requested by clinicians as an aid to diagnosis of virus pneumonia. It is also employed in the investigation of cases of haemolytic anaemia. A rapid and simple test for the presence of increased cold agglutinins was described[1] and its practical value and reliability confirmed.[3]

During a fifteen-month period, a further 861 "Garrow Tests" were performed in this laboratory, of which 495 were read both by Dr. D. H. Garrow and by a member of the laboratory staff. In a few instances, observers disagreed as to the recording of the result. Although in his original article Garrow made no differentiation between "negative (0)" and "negative (±)" results, for the purpose of this paper negative results were so subdivided in an attempt to analyze the importance of the "observer error" in the test.

## Materials and Methods

The tests were done on children who were in the wards or attended as outpatients in the Victoria Hospital for Children, Chelsea, London, England. They were suffering mainly from respiratory infections, and occasionally from other conditions in which increased cold agglutinins have been reported, e.g., glandular fever, leukaemia, infective hepatitis.

Blood was obtained by finger or ear-prick into a capillary pipette and then expelled into a little 3.8% sodium citrate in a small test-tube (3" x 3/8"). Ideally, 0.2 ml blood and 0.2 ml citrate were employed, but often it was difficult to obtain this amount of blood and it was found that the test worked equally well when quantities as small as 0.04 ml blood were used with an approximately equal amount of citrate. The tube was corked and rubbed against the "snow" in the ice-compartment of a refrigerator so as to cover the outside of the tube with ice crystals. After being left in the ice-tray for approximately 30 seconds, the tube was removed, held horizontally at the corked end, and rotated so that the citrated blood ran over the cold inner surface.

The presence or absence of agglutination visible to the naked eye was noted and recorded as follows:

| | |
|---|---|
| Coarse floccular agglutination | : positive (+) |
| No agglutination | : negative (0) |
| Fine granularity | : negative (±) |

The tubes were then left at room temperature and whenever possible examined later the same day by the other observer. Results were recorded independently without reference to the other's findings. When agglutination was noticed, its disappearance on warming was invariably confirmed.

* Received for publication December, 1960.

## Results

During the period 1st December 1958 to 29th February 1960, 861 tests were performed by the laboratory staff; of which 67 were positive and 794 were negative. The results were further subdivided as described above. Some of these tests were done on the same patient at different times during the period. A summary of the results is shown in Table I.

TABLE I

| | + | ± | 0 | TOTAL |
|---|---|---|---|---|
| TESTS | 67 | 104 | 690 | 861 |
| PATIENTS | 40 | 58 | 573 | 671 |

In Table II, the column headed + represents the number of patients who showed at least one positive result by either observer during the period. The column headed ± represents the number of patients who showed at least one negative (±) result by either observer during the period. The column headed 0 represents the number of patients who at no time showed a positive or a negative (±) result by either observer during the period.

TABLE II

| | + | ± | 0 | TOTAL |
|---|---|---|---|---|
| RESPIRATORY INFECTIONS | 38 | 52 | 495 | 585 |
| OTHER CONDITIONS | 2 | 6 | 78 | 86 |
| TOTAL | 40 | 58 | 573 | 671 |

Table III is divided into four parts to illustrate the fluctuation of the percentage of positive results during the period.

TABLE III

| | PATIENTS POSITIVE | PATIENTS TESTED | % POSITIVE |
|---|---|---|---|
| WINTER Dec, Jan, Feb, 1958-9 | 21 | 167 | 12·6 |
| SPRING & SUMMER Mar.- July (incl.) 1959 | 16 | 243 | 7·0 |
| SUMMER & AUTUMN Aug.- Nov. (incl.) 1959 | 0 | 130 | 0 |
| WINTER Dec, Jan, Feb. 1959-60 | 3 | 131 | 2·3 |

## Observer Error

Of the 861 tests recorded, 495 were read both by a member of the laboratory staff (RWW, PR or AK) and by Dr. D. H. Garrow (DHG). Of these, 453 tests were in agreement and the other 42 results showed disagreement. The results are shown in Table IV.

TABLE IV

| | AGREEMENTS | | | DISAGREEMENTS | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| LAB. STAFF | 0 | ± | + | 0 | ± | ± | + | + | 0 |
| D.H.G. | 0 | ± | + | ± | 0 | + | ± | 0 | + |
| Nº OF TESTS | 380 | 37 | 36 | 6 | 26 | 4 | 6 | 0 | 0 |

One member of the laboratory staff (PR) consistently obtained a high number of negative (±) results which, although often deemed to be negative (0) on retesting by either RWW or DHG, were recorded as PR read them. This fact increases the number of negative (±) results obtained in the period and, of course, increases the "disagreement" figures. The fine granularity observed in these cases, and in other cases where disagreement took place between observers, however, was only visible to the naked eye on very careful scrutiny. On the other hand, a positive result was usually clear-cut and obvious. In only 2% (10 of 495) cases was there difference of opinion in interpretation between negative (±) and positive results, and in no instance was there disagreement between negative (0) and positive results.

## Conclusions

From the data detailed above, it is permissible to deduce:

a) That there was a much higher incidence of positive results in the winter of 1958-59 than in the following winter. The raised incidence at the end of 1958 is accounted for by the occurrence of an outbreak of virus pneumonia (Garrow, 1959) and it is reasonable to consider that the continued recording of positive results in the first half of 1959 may be attributed to a continuation of the outbreak which appears not to have died out until the summer of that year.

b) That the source of observer error in this test lies mainly in the differentiation of negative (±) and negative (0) tests.

c) That the value of the test is affected only in a very small degree by the observer error.

## Summary

A survey of results of the Garrow test, a rapid test for increased cold agglutinins, has been analyzed. These results show that the phenomenon of increased cold agglutinins is relatively infrequent, and that the "observer error" of the test has a negligible influence on the accuracy of the results.

## Acknowledgments

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# SALMONELLA READING—A REPORT OF A HUMAN INFECTION*

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**Salmonella reading,** which was first isolated from the water supply of Reading, England,[1] is not ordinarily a causative agent of disease in human beings. The organism is found frequently as a contaminant of fish meal used in animal foods. The organism has also been found associated with poultry.[2,3] Drachman, et al[4] reported more than 300 human infections throughout the United States for the period, January 1956-July 1957.

Since this case was not included in the cases reported by Drachman et al, it is presented below as the first known proven case of *Salmonella reading* infection occurring in a human being in West Virginia.

## Case Report

A two year moderately ill boy who did not live on a farm was admitted to Broaddus Hospital on 2/18/57. The parents stated that the boy had bloody diarrhea of two days duration. He had loss of appetite, a reduced urinary output but had not been vomiting and was taking fluids well until the day of admission. The boy was found in a fair state of hydration although he had a mild respiratory infection. The abdomen was slightly distended with gas but there was no tenderness or any masses. Stool cultures and sensitivity tests were done on admission and the patient was started on cremomycin and oxytetracycline along with symptomatic measures. After the patient was started on this regime he continued to have 6 to 8 soft stool specimens daily. When it was found that the patient had a *Salmonella sp.,* he was started on chloramphenicol and streptomycin and two days later the stool specimens decreased to 1 or 2 a day. Stool cultures and sensitivity tests were done. The results can be seen under Laboratory studies. The patient's temperature chart is shown in Figures I to III. The remaining course of hospital stay was uneventful and the patient was released on 3/2/57. The stool at the time of discharge was negative for *Salmonella sp.* and the parents were instructed to continue antibiotics at home.

## Family History

All members of his family were apparently well and past history of the patient did not reveal any recent colds, infections or other diseases. There had been, however, an episode of diarrhea in the family approximately a year before this admission.

## Bacteriological Studies

**Salmonella reading** is a motile, gram negative rod, which occurs singly and in pairs, and grows well on most routine (ordinary) media. When the specimen was received in the laboratory, it was put into Selenite F broth and also on Eosin Methylene Blue (E.M.B.) and Salmonella-Shigella (S.S.) agars. This is routinely done on all stool cultures. Growth occurred on the E.M.B. and S.S. agars which indicated that it may be a possible pathogen (by its biochemical characteristics on these mediums).

* Received for publication July 1960.
** Assistant Director of Clinical Laboratories, now at Lee Memorial Hospital, Fort Myers, Florida.
*** Pediatrician—now at Graduate School, Univ. of Pa. Schl. of Med., Phila., Pa.

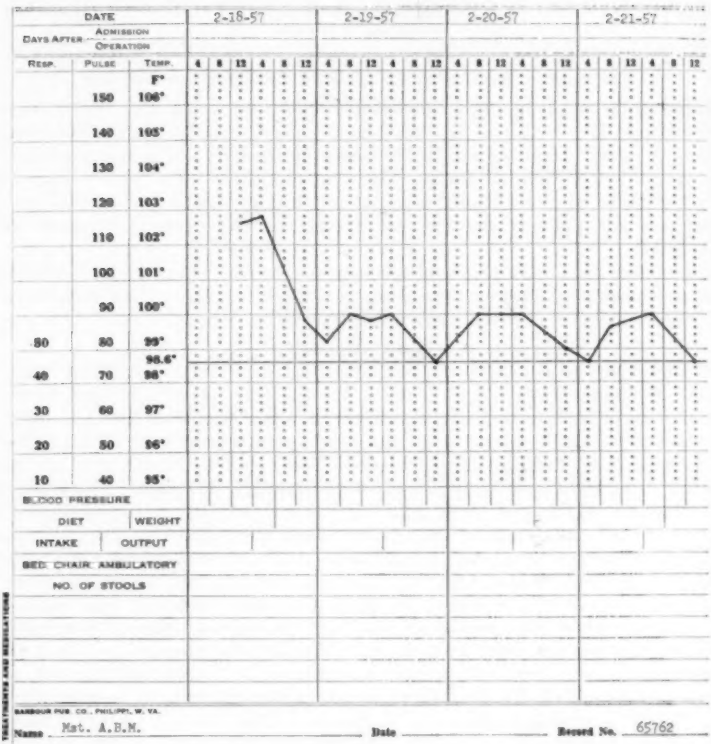


**Figure 1**

The suspicious colonies were then put to Russell's Double Sugar (R.D.S.), S.I.M.,* Citrate agar and Urea Broth.

After observing the reactions which occurred on the previously mentioned media, the organism was put through the sugars, and the following reactions were found in 24 hours

| | |
|---|---|
| Lactose | No change |
| Salicin | No change |
| Saccharose | No change |
| Xylose | Acid and gas |
| Dextrose | Acid and gas |
| Maltose | Acid and gas |
| Rhamnose | Acid and gas |
| Mannitol | Acid and gas |
| Dulcitol | Acid and gas |

* $H_2S$, Indol and Motility medium.

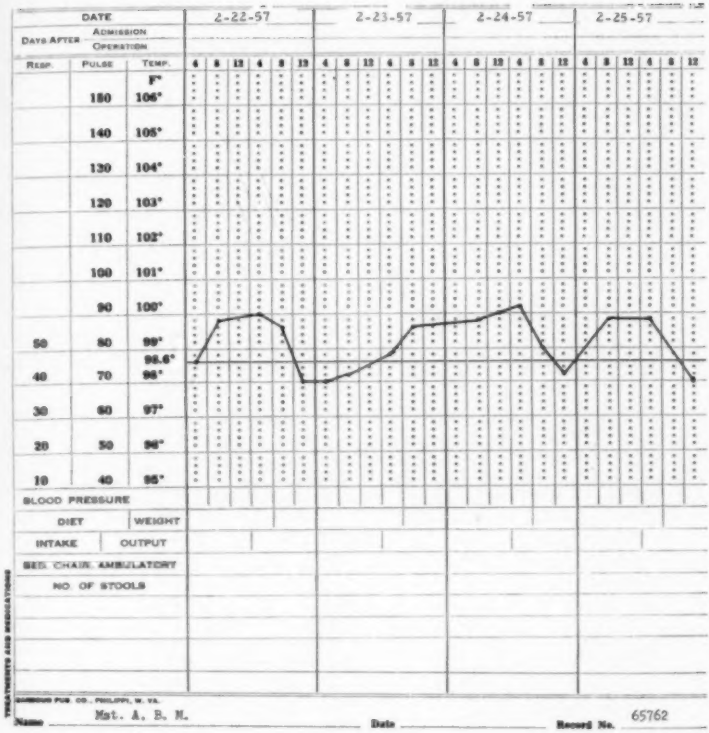


**Figure 2**

The organism was also found to be motile, produced $H_2S$, was indol negative, did not change urea, and utilized citrate as a sole source of carbon.

It was now known that the organism was a pathogen and was sent to the State Hygienic Laboratory for confirmation and serological studies.

## LABORATORY STUDIES

1. Complete Blood Count 2-18-57 (on admission)

White Blood Count — 7,100
Red Blood Count — 4,700,000
Hgb. — 11.5 Gms. — 81%
C. I. — 0.86
Granulocytes - - - - 61
Band Cells - - - - 24
Segmented - - - - 37
Eosinophils - - - - - - 1
Lymphocytes - - - - - 37
Monocytes - - - - - 1

Monocytes - - - - 1
Thrombocytes — 6 to 10 per oil immersion field.

2. First Urinalysis 2-18-57 (on admission)
Color — Pale yellow
Sp. Gr. — 1.016
Reac. — Acid; pH — 5
Sugar — Negative
Albumin — Ft. trace
W.B.Cs. = 10 — 30/HPF with occas. sm. clump.
R.B.Cs. = 5 — 10/HPF
Mucus — Large amount
Ep Cs.
Squa. — Occas.
Renal — few

3. First Stool Culture 2-18-57 (on admission)
(a) Organisms isolated
1. *Salmonella sp.* (giving biochemical reactions of *Salmonella schottmuelleri)*
2. *Escherichia coli*
3. Slightly hemolytic *Staphylococcus albus*
(b) Sensitivity Test versus First Stool Culture
1. Versus *Salmonella sp.*

| | |
|---|---|
| Effective | None |
| Resistant | Chloramphenicol |
| | Oxytetracycline |
| | Tetracycline |
| | Streptomycin |
| | Sulfadiazine |
| | Neomycin |

2. Versus *Escherchia coli*

| | |
|---|---|
| Effective | Chloramphenicol |
| | Oxytetracycline |
| | Tetracycline |
| Slight effective | Streptomycin |
| | Neomycin |
| Resistant | Novobiocin |
| | Matromycin |

4. Second Urinalysis 2-21-57
Color — Slightly cloudy, yellow green
Sp. Gr. — 1.023
Reac. — Acid; pH — 5
Sugar — Negative
Albumin — Negative
W.B.Cs. — Occas.
Mucus — Small amount
Ep. Cs.
Squa. — Few
Renal — Few
Crystals — Amorphous Urates

5. Second Stool Culture 3-23-57
   (a) Organism isolated
      1. *Salmonella sp.*
      2. Non-hemolytic *Streptococcus*
      3. Non-hemolytic *Staphylococcus albus*
   (b) Sensitivity Test not done

6. Third Stool Culture 2-27-59
   (a) Organisms isolated
      1. Non-hemolytic *Streptococcus*
      2. *Pseudomonas aeruginosa*
      3. No *Salmonella sp.* isolated

   (b) Sensitivity Test versus Third Culture
      1. Versus *Pseudomonas aeruginosa*

| | |
|---|---|
| Effective | Chloramphenicol |
| Slight Effective | Alba- penicillin |
| | Streptomycin |
| | Tetracycline |

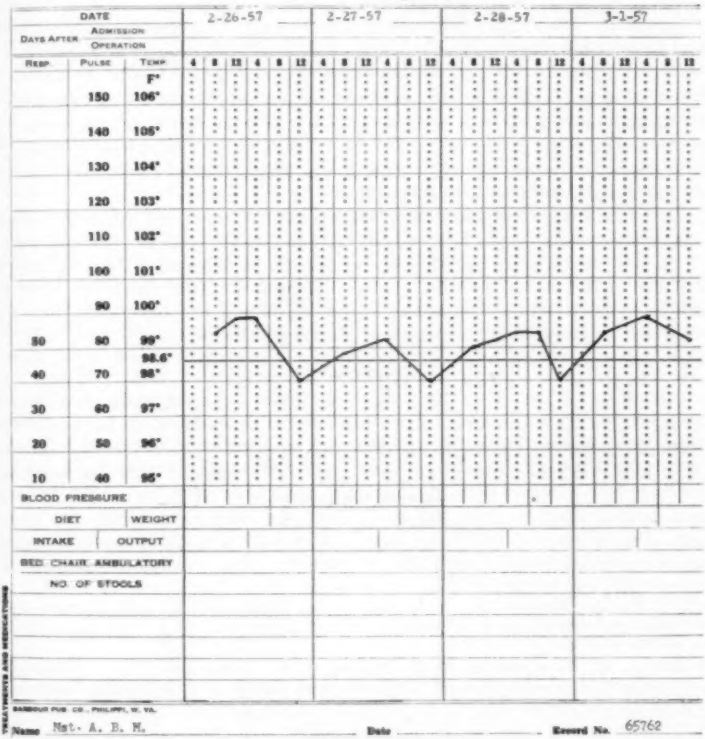


Figure 3

Resistant — Oxytetracycline
Matromycin
Furacin

2. Versus non-hemolytic *Streptococcus* — Not done

7. First Febrile
Agglutination Test 2-22-57*
Typhoid O — 1:40
Typhoid H — No agg.
Paratyphoid A — No agg.
Paratyphoid B — No agg.
Proteus OX — 19 — No agg.
B. abortus — No agg.

8. Second Febrile
Agglutination Test 3-2-59*
Typhoid O — 1:40
Typhoid H — No agg.
Paratyphoid A — 1:20
Paratyphoid B — 1:40
Proteus OX — 19 — No agg.
B. abortus — No agg.

* Using Lederle Febrile Antigens.

## Discussion

When it was found that the organism gave the biochemical reactions of *Salmonella schottmuelleri*, it was sent to a reference laboratory for the purpose of confirmation and also to let the State Health Department know that there had been a *Salmonella sp.* isolated. The State should always be informed of this from an epidemiological point of view.

It should be pointed out here that the reason we are credited with isolating this organism *(Salmonella reading)* is that we used the reference laboratory. Had we not sent the organism to the reference laboratory for serological typing, we would have thought it was a *Salmonella schottmuelleri* (and that would have been that). We would like to emphasize here that the State Laboratory be used more often as a reference laboratory.

## Summary

A case history has been presented in which a *Salmonella reading* was isolated from a human source. The only reason this organism is known to be a *Salmonella reading* is that the organism was sent to a reference laboratory for serological typing. The use of the State Laboratory is hereby advocated.

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# CREATIVE SUPERVISION—ITS GOALS†

## (Methodology of Accomplishment)

By WENDELL Y. SEVERSON, B.S., M.A., M.T. (ASCP) *Technical Director, School of Medical Technology Providence Hospital, Washington, D.C.*

Improvement is one of our natural professional goals.* It is the result of a creative process. In matters of mind and ideas it involves the organization of subjective life. As such, improvement is a matter to be encouraged, guided, and guarded (SUPERVISED).

How may it be done—as self-supervision or in supervision of others?

First, **understand the paradox of creativity.** Certainly new ideas are produced in the mind. But in creativity the idea makes, moves, and unifies the man. Thus it was not Goethe who created Faust but Faust who "created" Goethe. The idea and expression of relativity "sought" and "found" Einstein. The benzene ring "found" von Kekule. A man becomes the expression and servant of his idea. He is aware of significance to disclose itself. He has an open mind, receptive, ready to be "captured." This is the "dramatics" of creativity.

Second, **Make an Atmosphere, Climate, Environment, and Attitude Favorable to Creativity.** Encourage traffic and expression of ideas. ENRICH YOUR WORKING ENVIRONMENT with literature, films, discussions. Allow some amount of time during the working day to be unpressed by routine activity. Devote this planned time to creative work, to some improvement. Take time, make plans to grow, to improve. Think. Cultivate a mental life.

Third, **Stimulate Significant Purpose.** Cultivate vision. Make it relevant to the work at hand. You wish to master something. Is there nothing here to master? Nothing here unknown? No adventure? No danger? No hardship? You like change, novelty, excitement. You want recognition. Are there no opportunities for this here? These are some of the powerful reasons why we do what we do and think what we think. These are some of the mainsprings of accomplishment.

Both vision and significant purpose lead a man to peer into the unknown and ask questions: "What causes this?" "Why does this happen?" "What would happen if . . . ?" Significance and vision are mental products. Again, cultivate a mental life.

Vision and significance question what we do now and ask: "Is there a better way?" "A shorter way?" "An easier way?" "Can we do it this way?" "Can we add this?" "Eliminate this?" "Modify that?" "Reverse this?" "Substitute that?" Vision and significance would try to answer all these questions and many more in the effort to improve.

Fourth, **Appreciate.** This is creative. Appreciate your work. Appreciate that there is much for you to learn. You might make a contribution. There's room for you to grow in. You do not need to be a physician, or a Ph.D., or a professor, to improve procedures or to discover. Quite a few of the great ones were amateurs in the line of their discoveries.

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* In Dr. Leonard A. Scheele's address, "Year of the Turning Point: 1958" before the 26th annual convention of the ASMT, Milwaukee, Wis., June 1958. See Am. J. Med. Technol. July-Aug. 1958.

† Received for publication June 1960.

Joseph Priestly, discoverer of oxygen, was a parson. William Herschel, discoverer of his comet, and of the fifth moon of Saturn, and of the planet Uranus, was a musician. Gregor Mendel was a monk who taught Greek and Mathematics. Leeuwenhoek, who invented the microscope, was a linen merchant and janitor of the Council Chamber of the Sheriffs of Delft. Jean Henri Fabre, who wrote unrivalled observations of the life of insects, was self-taught. Heinrich Schliemann, discoverer of ancient Troy, was a business man. These all were amateurs in the lines of their discoveries. They made their living at other endeavor. But they discovered because they had vision. Their minds were open to ideas. They allowed themselves to appreciate. They allowed themselves to be captured by their creative ideas.

## SYMPOSIUM
## EXPERIMENTAL CANCER RESEARCH

# VIRUSES AND THE CAUSES OF CANCER*

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The notion that cancers may be caused by viruses has been a subject of controversy for more than 50 years. There is no evidence that all the diseases that we call cancer are caused by viruses, and there are many problems related to those tumors in animals that we know to be casually related to specific viruses. Some of these problems are linked with our inability to define completely the terms virus and cancer, other problems are related to our understanding of causation. I propose to tell you something about these problems so that you can form your own opinion of what is meant by the idea that viruses can cause cancer.

At present, a virus can be defined as an exceptionally small nucleoprotein-containing particle that can move from one cell to another, that can penetrate cells, and that can increase in number only by utilizing the metabolic machinery of the cell. This definition includes a remarkably heterogeneous group of things. There is some reason to believe that even such normal components of the cell as the gene and microsome might fit this definition. Some viruses are round blobs, others are rods, and some even have tails. They range in size and complexity from bacteriophages that are as large and complex as the more specialized bacterior to viruses that may function as single nucleoprotein molecules. Their diversity does not end at the molecular level. Variation among nucleoprotein molecules is presumed to be solely responsible for all variation in living organisms. A single deviation in the structure of a viral nucleoprotein molecule might be sufficient to establish a new virus and a new disease entity.

Because of the variety of things that fit our definition of virus, it is understandable that different viruses affect tissues in different ways. This is evident even in the more common types of viral infection. In infectious hepatitis and poliomyelitis the affected cell is simply destroyed, but in diseases resulting from infection with other viruses, cell destruction is partly balanced by cell proliferation. The pox lesions resulting from infection by herpes or vaccinia viruses are formed of both dying *and* proliferating epithelial cells. Measles and infectious mononucleosis are diseases in which the proliferation of lymphoid tissue can predominate. In the latter disease, proliferation is sometimes sufficiently extensive to be confused with leukemia. There are still other viral diseases in which cell proliferation exceeds cell destruction, and in which the proliferating cells persist for long periods of time. Diseases of this type are relatively common in animals. Leukosis and fowl pox in birds, fibromatosis and myxomatosis in rabbits, and venereal warts in dogs are all examples of proliferative response to viral infection. In man perhaps the best example of such a lesion is the cutaneous wart which is certainly a tumor, although limited in extent.

The proliferation of cells is not an uncommon tissue response to stimuli of various types other than viral infection. Many diseases of man are character-

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ized by modulations of cell proliferation and destruction. Some are the result of chemical injury, for example arsenical dermatitis; some, like the keloid, result from simple trauma, and some are the consequence of infection by micro-organisms far less exotic than the viruses. One of the earliest records of disease in man is a carving in stone by a pre-Incan Indian, depicting a victim of the tumor, *verruca peruana*. This tumor, common in Peruvians even today, is composed of cells that are proliferating in response to infection with the bacterium *Bartonella bacilliformis*. If at some later date it becomes important to determine the man that first described a tumor caused by micro-organisms, the pre-Incan sculptor of the figure should be given much consideration.

Although some proliferative diseases in man like *verruca peruana* may be fatal, thus far we know of no tumors in man that are caused by micro-organisms that progress beyond the stage of local growth. But in animals there are proliferative lesions caused by viruses and by other agents which spread to many organs and do not inevitably regress. The Shope papilloma is a tumor that develops on the skin of rabbits infected with a virus called the papilloma virus. The tumor usually behaves much like a human wart, but on occasion the warts progress to form tumors that have all of the characteristics of cancer in man! The progression of the rabbit wart to cancer occurs infrequently in infected wild rabbits but occurs commonly in infected domestic rabbits and in infected newborn rabbits of either species. This emphasizes that factors in the host play an important role in determining the ultimate behavior of a tumor.

Recently, an even more remarkable viral proliferative disease has been discovered that can affect mice, hamsters, rats, rabbits, and perhaps other rodents. The virus that causes this disease is called the polyoma virus because, unlike the Shope papilloma virus, the polyoma virus causes cells of many organs to proliferate if a large enough dose is inoculated to spread the virus throughout the body. Polyoma virus, like many other viruses, causes cells to degenerate as well as proliferate. Degeneration in the infected cells is characterized by rather specific nuclear alterations and inclusion bodies which lead to the ultimate destruction of the cell. Within the degenerating cells one can find many particles that have the structure of viruses. These cells do not proliferate. However, it is possible that cell destruction by the virus may be necessary for the proliferative phase of this disease, because focal areas of cell proliferation follow the degenerative changes in many of the affected organs. Oddly, it is difficult to find virus in the proliferating cells. Some foci of proliferating cells grow very slowly or cease growing altogether and regress; some can be found only in the most careful microscopic examination, but other foci grow until they have attained immense proportions. However, even the massive tumors generally do not have all the attributes of cancer as we know the disease in man. Unlike most cancers, the tumors retain an orderly circumscribed growth pattern involving only the organ in which they arise. They are responsible for the death of the animal because they consistently occur as multiple tumors in vital organs and reach great size.

Before we consider the problem of whether these lumps of proliferating cells are cancers, it would be valuable to outline ways that a virus might bring about this proliferative response.

There is no doubt that proliferation occurs in tissues parasitized by the virus, but one cannot equate infection and tumor formation in a particular tissue. Some tissues in which the virus grows do not develop tumors, and conversely, not all tumors show evidence of virus infection throughout their existence. Furthermore, there is some evidence that the particular cells that are parasitized by the virus are not the cells that proliferate. It is probable that the growth advantage gained by the tumor cells over normal cells comes about in a more complex manner than the simple infection of cells by the virus.

Figure 1 illustrates some of the ways that this might occur. What we know of the course of most viral infections is illustrated in the center of the sketch. When a virus enters a cell, its protein coat is separated from the nucleic-acid core and may not enter the cell at all. The nucleic-acid fraction is capable of reconstructing new viruses including their protein coats from materials supplied by cellular metabolism. This process may take minutes or it may take months and may or may not result in the destruction of the cell, but ultimately new virus is released to infect other cells. Such events undoubtedly occur with the tumor viruses, but, while the cell is host to the viral nucleic acid, other things happen that stimulate excessive cell growth.

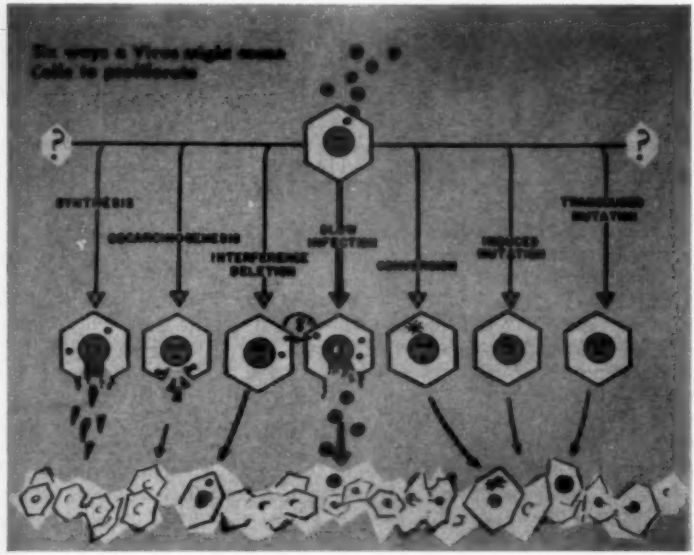


**Figure 1**

The theory of transduced mutation proposes that genetic material from infected cells or the nucleic acid of the virus itself is carried over and incorporated into the chromosomes of newly infected cells. The newly infected cells then would be mutants with an enhanced capacity to proliferate. Cellular genetic material can be transported and incorporated in new cells as demonstrated by the phenomenon of lysogeny in bacterial viruses, but as yet there

is no firm evidence that the transport of genetic material can stimulate cell proliferation in either bacterial cells or cells of higher organisms.

An alternative theory proposes that the virus induces a mutational change in the cell without entering its genetic structure. Theoretically, the impact of virus in the cell alters native genetic material, consequently releasing the cell from growth control of the host. However, many agents known to induce mutational alterations in chromosomal structure do not stimulate cells to proliferate. Furthermore, most proliferative lesions induced by viruses do not show detectable chromosome alteration.

The virus might alter elements in the cell other than the heritable genetic apparatus. The diversion or conversion of cellular proteins or nucleic acids to viral use might stimulate the cell to proliferate. Such a theory falls in line with the delection hypothesis proposed by some biochemists and immunologists to explain the oncogenicity of the diverse group of chemicals known to induce tumors.

It is also possible that viruses might interfere with homeostatic mechanisms that exist between related cells for the control of growth. We know that such mechanisms exist, for cells in tissue culture that are freed of their relationship to other cells grow with remarkable rapidity and lose characteristics of differentiation. Interference by virus with these intercellular homeostatic systems could stimulate cell growth either by blocking or depleting natural inhibitors of growth or by enhancing the inherent ability of cells to multiply.

On the other hand, the virus may not be directly responsible for cell proliferation at all. Its presence may simply make the cell more susceptible to other factors in the environment that in turn stimulate cell proliferation. The virus may simply open the door, allowing other factors to act. The theory of cocarcinogenesis, in which an initiating factor prepares the way for a second factor that promotes the proliferation of cells has been well established for many chemical carcinogens. If viruses act in a similar system, it will be necessary to determine whether they act as initiators or promoters. Both endogenous and exogenous factors that might have cocarcinogenic action in viral oncogenesis are well known. Age, species susceptibility, endocrines, chemicals, trauma, and radiations have all been found to influence the onset and character of various viral proliferative diseases.

One final theory regarding virus as a stimulus in cell proliferation deserves attention. The destruction or damage of a cell might alter the function of either the cell or the virus to the extent that new particles alien to both cell and virus are synthesized, and these in turn might set the cell off on a proliferative spree. There is evidence for this type of reaction in other diseases. Only recently, auto-antibodies, which are alien proteins derived from altered host cells, have assumed significance in thyroiditis and glomerulonephritis. In tumors, other products of cell-virus interaction, for example the enzyme interferon, could conceivably stimulate cells to proliferate.

The various theories invite questions that must be answered if we are to understand proliferative phenomena. In some theories it is necessary that all or a part of the virus remain in the cells if growth is to be continuous. Detecting or unmasking this hypothetical viral particle has become a real challenge to the virologist. Other theories indicate that once the virus had

altered a cell the cycle of unrestrained growth could continue without further help. Virus particles would not necessarily remain in the tumors they induced, and all chance of their detection would be lost unless the earliest stages of the disease were studied or unless the viruses continued to grow as inactive passengers in the proliferating cells. Alternatively, it is possible that the cells infected by viruses are not the cells that proliferate. Cells adjacent or far removed from parasitized cells may be stimulated to grow by intermediate products of infection. The facts that we now know are not sufficient to secure any of these theories. It is probable that none of them are entirely correct. We attempt to prove or disprove them because our efforts enable us to find new facts, ask more questions, and construct new theories. All these efforts contribute to our understanding of diseases that are characterized by uncontrolled growth. It is not certain, however, that comprehensive knowledge of these processes will lead to a complete understanding of the diseases we call cancer. The term cancer implies far more than the simple uncontrolled growth of cells.

Cancer, like virus, is extremely difficult to define. A practical definition would necessitate defining the term tumor and defining cancer as a particular class of tumor. A tumor can be defined as any abnormal growth of tissue. However, tumors have no qualities of either behavior or structure that cannot be found in non-tumorous tissues. In normal regenerating tissues, growth may be as rapid as growth in any tumor. In the embryo, the destruction and invasion of tissues during the formation of the placenta and reorganization of embryonic structure are equivalent in every way to the behavior of a malignant tumor. The deviations that occur in both structure and chemical composition of tumor cells are only quantitatively different from normal tissues. Furthermore, they can occur in other tissue reactions and may be inconspicuous in the most malignant tumor. It is necessary, then, to separate tumors from normal tissues and tissues of other abnormal reactions in terms of *quantitative* exaggerations in behavior and structure.

This permits a reasonable definition of cancer, for a tumor may be considered cancer if quantitative changes in its growth and behavior are sufficient to destroy the host. This does not mean that every tumor that kills is cancer, for tumors kill in many ways. Some tumors kill only because they occur in a vital area, others kill because they attain great size, and still others kill because they adversely affect the metabolism of the host either through excessive nutritional demands or through elaboration of toxic excretory products. The term cancer is usually restricted to tumors that are lethal because they have acquired the ability to invade and destroy normal tissues or because they have acquired the ability to spread and grow in sites distant from the initial focus of growth. The detection of these attributes in a tumor and the knowledge of the potential behavior of tumors of like morphology enable the pathologist to make a diagnostic prediction. A pathologist calls a tumor cancer if such behavior is evident in a tumor or if his past experiences indicate that tumors of similar type and site are capable of such behavior. However, it is important to remember that cancerous tumors are only quantitatively different from non-cancerous tumors. We cannot assign every tumor to a separate category of innocence and malignancy because there are infinite grades of tumor behavior just as there are infinite varieties of personal behavior. The word cancer, like the words good and bad, is a relative

term with many meanings that depend on gradations of aggressive and destructive behavior. Fortunately many tumors stand at either end of this scale of behavior and remain either clearly innocent or malignant. This is well illustrated in two tumors, the leiomyoma and the leiomyosarcoma, that arise in the uterus of human beings. We do not know the causes of either of these tumors, but their behavior is remarkably different. Uterine leiomyomas are composed of circumscribed nodules of poorly differentiated cells of mesodermal origin. These tumors are often multiple; they may grow rapidly, and they frequently reach great size. Yet, they do not invade tissues or metastasize, instead they grow as well-defined, ever expanding, circumscribed nodules. The cells of the leiomyomas differ from normal cells only in those qualities which reflect a more rapid rate of growth. In contrast to the leiomyoma, the leiomyosarcoma of the uterus is a malignant tumor that arises from the same type of cell but can behave in an entirely different manner from its onset. The sarcoma kills because it invades and destroys both the uterus and adjacent organs and because it consistently metastasizes to distant organs. It can do this long before reaching the size attained by the average leiomyoma, and need not show the more orderly growth characteristics of its innocent counterpart in any phase of its development. Here then are two types of tumor related both in site and cell of origin, but easily distinguished by their activities. However, this is not the whole story; some cells in a few of the leiomyomas may also acquire the ability to invade and destroy tissues, progressing to this malignant stage only after the tumor has reached large size. The quantitative variations in the behavior of tumors are not static changes that differ only between tumors, but they are dynamic changes that can occur within a single tumor during its growth. The natural history of many types of tumors is one of progressively acquired new characteristics in the direction of more malignant behavior. Cancer, then, is any tumor that from its onset *or* during the course of its history has acquired the ability to invade and metastasize and by this process destroy the host.

Where do the virus-stimulated lumps and bumps of proliferating cells fit into this concept of cancer? The initial behavior of the tumors induced by most tumor viruses is somewhat analogous to the behavior of the leiomyomas of man. Both are composed of cells that seem to possess no malignant characteristics other than the singular ability to proliferate rapidly. But just as leiomyomas and many other predominantly benign tumors can become cancerous, a few of the viral tumors also can become cancerous destroying tissues and metastasizing just as cancer does in man. The problem of how this progressive malignant change comes about in virus-induced tumors has not been solved. Is the change related to the virus? Perhaps it is, and perhaps it comes about through some of the mechanisms that I illustrated a a moment ago. But in the polyoma-virus-induced lesions, the progressive acquisition of malignant behavioral characteristics cannot be correlated with the presence or absence of virus. The polyoma tumors may lose all evidence of possessing virus long before reaching this stage. Furthermore, tumors induced by chemicals and radiations also can be composed of simple, proliferating cells initially, and can acquire more vicious traits long after the elements that initiated their development have disappeared. The process of progression through which cells acquire malignant characteristics may be unrelated to the viral, chemical, or radiological stimuli that initiate prolifera-

tion. Progression may be analogous to embryonic development, although there is no evidence that the two are related. Development of the embryo is initiated by fertilization of the egg, but the orderly sequence of events leading to the complete formation of the individual is influenced and controlled by many factors. One can say that fertilization of the egg causes development of the embryo, just as one can say viral proliferative mechanisms cause the development of some cancers, but few students of embryology or oncology would be satisfied to let matters rest with these simple statements.

The cause of a cancer is not a single problem; it is many problems. It concerns the multitude of factors that start cells to proliferate; it concerns the factors that enable them to continue to proliferate, and it concerns the factors that enable some proliferating cells to gain the ability to invade, metastasize and destory the host. These factors, whether they be particular viruses, radiations, chemicals, or agents endogenous to the organism, cannot cause cancer without a cell to play the major role in this disease. The ultimate cause of cancer lies within the cell, not within the many etiologic agents involved. These cellular mechanisms are still unknown. When they are found it may be a simple matter to deal therapeutically with the disease. The value of studying etiological factors such as animal tumor viruses lies in the contributions that they may make in revealing intracellular mechanisms of carcinogenesis.

# COMPARATIVE ASPECTS OF CANCER IN ANIMALS*

By J. R. M. INNES, Sc.D. (Cantab.), Ph.D., M.R.C.V.S.
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Cancer is a disease which occurs ubiquitously throughout the animal kingdom including apes and monkeys, although for years the latter were though to be highly resistant. Differences in cancer between animals and man certainly exist, but these are not fundamental; they are closely related to the well-known divergent susceptibilities of the varying animal species to the occurrence of different types of cancer, and thus to the unmistakable higher or lower incidence compared with human data, according to involvement of different organs or tissues. Possibly the over-all morbidity rate of malignant disease is about the same in all domestic species as in man if animals were allowed to survive their natural life span. Some organs of animals are more prone to be affected with cancer than in man—for example, the testes and mammary glands of the dog, while other organs are much less so, such as the mammary glands of the cow and stomach of all species. Domestic animals have varieties of cancer which are peculiar to certain species on even breeds; for example, eye cancer in cattle (Hereford), osteogenic sarcoma of long-legged dogs, osteogenic sarcoma of the mammary glands of dogs; leukemia in Scottish terriers, although probably much more data are required to be certain these are true facts. There are forms of cancer of certain organs which are both frequent in, and common to, both man and animals, and in which causation can only be presumed to be the same. Cancer of farm animals, killed for human consumption, and at an age when they are relatively young, is an economic problem. Natural cases of cancer in the small domesticated animals are common enough, so that much to great advantage might have been learned from controlled clinical trials by X-irradiation or chemotherapeutic agents.

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For further information see Innes, J. R. M: Malignant diseases of domesticated animals. *In:* Cancer (Ed. R. W. Raven), Vol. 3, pp. 72-115, London: Butterworth, 1958.)

* Received for publication September, 1960. An abstract of a talk made before the 28th Annual Convention of ASMT, Atlantic City, New Jersey, June 1960, Cancer Panel.

# OCCUPATIONAL CANCERS WITH SPECIAL REFERENCE TO OCCUPATIONAL CANCER HAZARDS TO LABORATORY PERSONNEL*

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An increasing amount of epidemiologic, pathologic, clinical and experimental evidence acquired during recent decades supports the view that many human cancers are directly or indirectly related to contacts with certain specific environmental physical and chemical agents to which the population becomes exposed for occupational, medicinal, dietary, or other environmental reasons (table 1). Among the numerous and diverse claims advanced concerning the causation of human cancers, these data provide indeed the only factual and well established information in such matters. The data on hand strongly indicate that the same agents which cause occupational cancers most probably are also responsible for similar cancers occurring among members of the general population because of the dissemination of the occupational carcinogens into the general human environment as parts of industrial pollutants of the air, water and soil and as constituents or impurities in consumer goods. The majority of cancers of the skin for instance, can be traced to exposures to recognized environmental carcinogens, such as sunlight, ionizing radiations, coal tar and pitch, lubricating and cutting oils derived from petroleum, and arsenicals. The development of many cancers of the lung seem to be related to exposures to known environmental carcinogens, such as polycyclic hydrocarbons contained in coal tar fumes, coal pitch dust, cutting oil mists, exhaust fumes from automobiles, dusts and fumes containing arsenicals, chromates, nickel compounds, asbestos, and radioactive chemicals. Similar relations to environmental carcinogens prevail for cancers of the bladder, bones, blood forming organs, alimentary tract and liver. There exists a considerable number of additional chemicals which must be suspected of exerting at least in part, similar carcinogenic effects on man upon proper contact. However, at present the evidence incriminating these compounds which mostly form newly added constituents of our modern industrial economy with which large parts of the population often have frequent and intimate contact is not sufficiently adequate or too fragmentary for definitely incriminating them at this time in the production of cancers in man (table 2). They must be considered, however, as potential human carcinogens until their innocuousness to man can conclusively be established.

Since the most readily demonstrable and usually most intense exposures to the various occupational and industry-related carcinogens takes place in general among members of certain industrial worker groups, the majority of known cases of cancers caused by these environmental carcinogenic agents was observed in such population groups. However, the occurrence of occupational cancers has not remained restricted to specific worker groups engaged in the production, processing, handling

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**TABLE 1**
**Occupational Carcinogens, Their Route of Exposure and Their Target Organs**

| General Type | Specific Type | Route of Exposure | Target Organs |
|---|---|---|---|
| Chemical Carcinogens Organic Chemicals Aromatic, Polycyclic, Heterocyclic | Benzol | Cutaneous<br>Respiratory | Blood Forming Organs (Leukemia, Lymphosarcoma) |
| | Beta-Naphthylamine<br>Benzidine<br>4-Aminodiphenyl | Respiratory<br>Cutaneous<br>Alimentary | Urogenous Organs (Bladder, Ureter, Kidney) |
| | Auramine | | Lung? Intestine? |
| | Coal tar, Pitch | Cutaneous | Skin |
| | Asphalt, Tar Oil, Creosote oil, Anthracene oil, Lamp black, Lignite tar and Paraffin oil | Respiratory | Lung |
| | Synthetic hydrogenated coal oil and Tar (Bergius) | Cutaneous | Skin |
| | Shale oil and Paraffin oil | Cutaneous<br>Respiratory | Skin<br>Larynx |
| | Petroleum fuel oil, Diesel oil, Lubricating oil and Grease, Cutting oil, Carbon black, Asphalt, Tar, Coke Crude Paraffin oil | Cutaneous | Skin |
| | | Respiratory | Lung |
| Aliphatic | Isopropyl oil | Respiratory | Nasal sinus<br>Larnyx<br>Lung |
| | Mustard gas | Respiratory | Lung, Larnyx? |
| Chemical Carcinogens Inorganic Chemicals | Arsenic | Cutaneous<br>Ingestive | Skin, Nasal sinus, Lung, Liver |
| | | Respiratory | Lung |
| | Nickel | Respiratory | Nasal cavity<br>Nasal sinus<br>Lung |
| | Chromium | Respiratory | Lung, Nasal cavity, Nasal sinus |
| | Asbestos | Respiratory | Lung, Pleura |
| Physical Carcinogens Ionizing Radiation | Ultraviolet Radiation | Cutaneous | Skin |
| | X-Radiation | Cutaneous | Skin |
| | | Transcutaneous | Connective tissue<br>Bone<br>Blood forming organs |
| | Radioactive Radiation (Alpha-Beta-and Gamma Radiation) | Cutaneous<br>Transcutaneous<br>Respiratory | Skin<br>Connective tissue<br>Blood forming organs<br>Nasal sinus<br>Lung |
| | | Ingestive<br>Parenteral | Bone<br>Liver |
| Parasite Carcinogens | Schistosoma Hematobium | Cutaneous | Bladder |

**TABLE 2**
**Potential Environmental Human Carcinogens**

| Agent | Site of Tumor | Type of Exposure | Species | Types of Human Contact |
|---|---|---|---|---|
| Estrogens, natural, synthetic | Breast<br>Lymph nodes<br>Uterus<br>Hypophysis | Parenteral<br>Cutaneous<br>Oral | Mouse<br>Rat<br>Rabbit<br>Guinea pig | Manufacture<br>Dietary additive<br>Cosmetic ingredient |
| Carbon tetrachloride | Liver | Oral | Mouse | Industrial solvent Degreasing agent, Dry cleaning agent, Fire extinguishing agent, Manufacture Production of Freon, Grain fumigant, Extractive of oils |
| Chloroform | Liver | Oral<br>Parenteral | Mouse | Manufacture<br>Anesthetic<br>Solvent and extractive of oils, resins, rubber, waxes, iodine, alkaloids<br>Ingredient of lacquer, floor polish, cleaning fluid<br>Production of artificial silk, plastics |
| DDT | Liver | Oral | Rat<br>Dog | Manufacture<br>Insecticide<br>Food contaminant |
| Aramite | Liver | Oral | Rat, Dog | Miticide |
| Tannic acid | Liver | Parenteral | Rat | Ingredient of foodstuffs (fruits, wine, coffee, and tea)<br>Medicinal agent<br>Tanning agent |
| Polyoxyethylene (8) stearate | Bladder | Oral | Rat | Emulsifier in bread |
| Thiourea and derivatives | Liver | Oral | Rat | Citrus fruit preservative |
| Thioacetamide | Thyroid | | | Medicinal agent |
| Dulcin | Liver<br>Bladder | Oral | Rat | Manufacture<br>Sweetening agent |
| Diethylene glycol | Bladder | Oral | Rat | Antifreeze<br>Intermediate in explosive manufacture<br>Softener of lacquer, inks, wood stains, glue, textiles<br>Humectant, tobacco |
| 8-Hydroxyquinoline | Uterus | Vaginal | Mouse, Rat | Countraceptive, Disinfectant |
| Methylated naphthalenes | Skin | Cutaneous | Mouse | Vehicle of insecticides |
| Bergius coal oils, tars | Skin<br>Subcutaneous tissue | Cutaneous<br>Parenteral | Mouse | Manufacture<br>Fuel<br>Lubricants<br>Plastic production<br>Petrochemical |
| Beta-propiolactone | Skin | Cutaneous | Rat, Mouse | Virucide (Vaccine, Plasma, Room & Air Disinfectant) |
| Fischer-Tropsch coal oils, tars, waxes, greases | Liver | Cutaneous<br>Parenteral | Mouse | Manufacture<br>Fuel<br>Lubricants<br>Petrochemical |
| Aminotriazole | Thyroid | Oral | Rat | Herbicide |
| Light green, S.F. | Subcutaneous tissue | Parenteral | Rat | Textile dyes<br>Food and cosmetic dyes |
| Brilliant blue, F.C.F. | | | | |
| Butter yellow | Liver | Oral | Mouse<br>Rat | Food dye (Orient)<br>Gasoline and flare dye |
| Cellophane<br>Polyethylene<br>Polyamide<br>Bakelite | Subcutaneous | Parenteral | Rat | Film, Fiber, plastic-industrial manufacture & uses<br>Wrapping material<br>Medicinal agent |

**TABLE 2 (Cont'd)**
**Potential Environmental Human Carcinogens**

| Agent | Site of Tumor | Type of Exposure | Species | Types of Human Contact |
|---|---|---|---|---|
| Iron-Dextran | Subcutaneous tissue | Subcutaneous | Rat, Mouse | Anti-anemic drug |
| Beryllium | Bone | Parenteral<br>Respiratory | Rabbit<br>Rat | Metal alloy, X-ray tube, phosphor manufacture<br>Refractory vessels<br>Atomic energy production |
| Selenium | Liver<br>Thyroid | Oral | Rat | Soil contaminant<br>Coloring matter of glass, ceramics, paint, rubber<br>Metal alloys<br>Rubber accelerator<br>Photoelectric apparatus<br>Decolorizer<br>Fireproofing agent<br>Medicinal agent |

and using these industrial carcinogens. In fact, a few occupational cancers have been observed preferentially among members of certain professions, such as radiologists. Considering the fact that an effective therapeutic control of many cancers is still to be obtained, it is important to note here that the development of the great majority of occupational cancers can successfully be prevented by available precautionary measures. The most essential requirement preliminary to accomplishing a preventive control of occupational cancers is the proper awareness of the exposed individuals, of the existence, types and sources of occupational cancer hazards. It is the particular purpose of this presentation to alert technologists employed in medical laboratories to cancer hazards which may be associated to agents handled and activities pursued in such establishments.

1. **Benzene or Benzol.**—Benzol or benzene is a leukemiogenic agent which has caused leukemia among rubber cement workers, employed in rubber factories and canned food plants, painters, rotogravure printers, and chemical workers. Exposed persons may exhibit first aplastic hematic effects before hyperplastic and leukemoid manifestations appear. Skin contact with benzene or inhalation of benzene vapors sustained during laboratory operations requiring the use of benzene as a vehicle or diluent or as a solvent in the extraction of oils, fats and lipoids, or as an eluent of chromatographic studies might prove dangerous if occurring frequently. Such laboratory procedures, therefore, should best be carried out in a hood or with adequate exhaust ventilation. Laboratory workers routinely doing such work and having such contacts should be kept under periodic medical surveillance, including blood examination, for evidence of injury to the blood-forming tissues. Evidence of hematic damage is an indication of the inadequacy of the precautionary measures used. Injured individuals require often a prolonged period of medical care and absence from work for complete recovery. It is important from a clinical as well as medicolegal viewpoint that the leukemic phase of chronic benzene injury may become manifest several months after apparent recovery from a primary degenerative phase. Since benzene is retained and stored in the fat tissue of the bone

marrow, it may nevertheless be possible at such a time to demonstrate benzene in this tissue by chemical analysis. Such a test has been positive some months after cessation of exposure to benzene. The occupational origin of a leukemia can thereby be established.

2. **Aromatic Amines.**—Several aromatic amines, namely, betanaphthylamine, benzidine and 4-aminodiphenyl, have caused cancers of the bladder following a cutaneous and respiratory exposure among producers of these dye and rubber antioxidant intermediates, and among their users in the manufacture of azo-dyes and rubber antioxidants as well as among rubber workers. These chemicals are readily absorbed through the skin and are highly potent human carcinogens accounting for over 2,000 cases of bladder cancer among German, Swiss, English, Italian, American, Russian and Japanese dye workers. The total number of cancers caused by occupational and environmental agents (dietary, cosmetic) is doubtlessly considerably higher than this number because many of these cases were either not properly recognized concerning their specific environmental etiology, or were not reported. Since beta-naphthylamine has recently been demonstrated as an impurity in two formerly certified food-drug- and cosmetic dyes, namely, yellow AB and yellow OB, and since these dyes were until recently certified for external cosmetic use, the number of persons who became exposed for many years to beta-naphthylamine through the consumption of margarine, butter, cheese and cookies colored with these dyes, is indeed very high. It is, on the other hand, unknown whether any bladder cancers have resulted from such prolonged exposures.

Employees in medical laboratories may have contact with beta-naphthylamine, if this chemical is produced and/or used on tissue sections for the histochemical demonstration of certain proteinases. If in such procedures beta-naphthylamine containing solutions and containers are not handled with extreme caution so as to avoid undue contact particularly by their spillage on bench tops and floors, not only technologists, but also cleaning personnel may sustain an effective exposure to beta-naphthylamine. Because beta-naphthylamine does not elicit any acute or chronic toxic symptoms, such contacts may not attract any attention until after several years suddenly and without warning symptoms the appearance of blood in the urine and other urinary disturbances signify the presence of a bladder cancer. These cancers, like any other occupational cancer, may develop many years after cessation of exposure to the carcinogenic agent. The extreme degree of cancer hazard created by an occupational contact with beta-naphthylamine, which is measured in terms of micrograms when sustained over some months or years, is evident from the fact that the dye industries in Germany, England, Switzerland and in part also in the United States have discontinued for a number of years the production of beta-naphthylamine because of its extreme and technologically uncontrollable occupational cancer hazard. The demonstration of 2-amino-1-naphthol sulfate or glucuronate in the urine may serve at times as a diagnostic sign of an exposure to beta-naphthylamine.

Benzidine is of slightly lesser carcinogenic potency than beta-naphthylamine. Benzidine has been used for many years for the demonstra-

tion of occult blood in urine, feces and gastric content. From more recent experiences made in industry it appears likely that benzidine does not only cause cancer of the bladder but also of other organs, such as the intestine and lung.

Although the occurrence of bladder cancer among technologists of medical laboratories from contact with these aromatic amines has as yet not been reported, such sequelae have been recorded among laboratory workers in the chemical industry handling benzidine and betanaphthylamine, among cleaning personnel, and among laundresses washing work clothes contaminated with these aromatic amines. These observations indicate that distinct precautions and extreme cleanliness are necessary whenever carcinogenic aromatic amines are handled, processed, or used in a medical laboratory. Adequate protection demands that these chemicals be used only in a hood for avoiding contact with dust and vapors, and the contamination of rooms from spilled solutions. Since it has been alleged in the past years that males are mainly or only susceptible to the carcinogenic action of these aromatic amines, it may be mentioned that bladder cancers have experimentally been produced with betanaphthylamine in female dogs and that recent reports from U.S.S.R. and Italy noted their occurrence among female dye workers.

3. **Coal Tar, Pitch, Petroleum Oils, Paraffin, Asphalts and Carbon Blacks.**—Medical technologists, as a rule, have little opportunity for a frequent and intimate contact with the carcinogenic products of the distillation and combustion of coal, petroleum and related carbonaceous matter. It may be mentioned, however, that crude paraffin oils as well as insufficiently refined paraffin waxes are carcinogenic to the skin and lung. Histologic technologists, therefore, may be advised to avoid a frequent inhalation of fumes from heated paraffin waxes and should not adopt the habit of chewing paraffin plugs. Similar precautions should be applied to the use of mineral oil baths for chemical procedures. Burns caused by hot mineral oil or wax should thoroughly be cleaned to prevent retention of any oily or waxy matter in the injured tissue which might give rise to a subsequent development of a cancer.

4. **Arsenic, Chromium, Nickel, Asbestos.**—It is not likely that the working conditions and practices in medical laboratories are associated with effective exposures to the various carcinogenic minerals, such as arsenic, asbestos, chromium and nickel. These have resulted in occupational cancer hazards leading to the development of cancers of the respiratory organs, i.e., lung, larynx, nasal cavity and nasal sinuses, and, for arsenicals also, of the skin and liver. Effective exposures may occur, however, for those medical technologists who are attached to medical departments located on the premises and thus in the dust and fume zone, of chromate producing plants, nickel refineries, copper smelters using arsenic-containing ores, and asbestos mills and textile plants. The observation of a perforated nasal septum in a nurse employed in an American chromate plant attests to the actual existence of such exposure conditions for medical personnel working in industrial medical establishments located within the immediate neighborhood of such plants.

5. **Ultraviolet Radiation.**—Cancers of the exposed skin appearing in man, mice and rats after an excessive and prolonged exposure to solar radiation and ultraviolet radiation from mercury or carbon arcs and following the development of a precancerous actinic dermatitis, furnish definite proof of the carcinogenic action of ultraviolet rays. The carcinogenic action of this actinic energy upon the human skin accounts for the high frequency of cancers of the exposed skin among the inhabitants of our Midwestern, Southwestern, Western, and Southern States, including Florida, Southern California, and Hawaii. Technologists working in heliotherapy and ultraviolet therapy departments when sustaining excessive exposures to ultraviolet radiation, unless they observe the proper protective precautions, may develop occupational cancers of the skin. Most susceptible to the carcinogenic action of ultraviolet radiation is the skin of fair-complexioned individuals who do not tan upon exposure, but burn. Brunette whites show a somewhat higher resistance. Chinese, Japanese, Filipinos and members of related races are even more resistant than whites, while deeply pigmented Negroes appear to be entirely refractory to the development of solar cancer of the skin. Such environmental and occupational cancers are often multifocal and appear in a skin showing signs of a chronic solar dermatitis, also known as farmer's skin and sailor's skin, since such occupational conditions occur not infrequently among members of these population groups.

Individuals who display a special constitutional liability to solar cancers and become frequently exposed for occupational or other reasons to ultraviolet radiation, therefore, should wear adequate clothing, and hats with wide brims and might additionally apply protective creams to avoid cancerous sequelae.

6. **Ionizing Radiations.**—Medical technologists have been among the early victims of X-ray cancers of the skin following the introduction of roentgen-rays into medical practice in 1895. It was only a few years later that this sad experience was repeated in medical personnel handling radioactive substances for therapeutic purposes as well as in patients who had received excessive amounts of X-rays. Such occurrences were rather frequent among the pioneers of radiology, because the cancer hazard from exposure to ionizing radiation was not known or sufficiently appreciated during the early days. The actual number of victims of occupational radiation cancers is unknown. For several decades, cancer hazards from ionizing radiation for laboratory personnel remained restricted to radiations generated by X-ray tubes and given off by radium, radon, and mesothorium employed in diagnostic and therapeutic medical practice. It was only in more recent years that the growing use of electronmicroscopes, X-ray diffraction apparatus and television projection equipment added for certain types of laboratory personnel a few new sources of radiation hazards resulting from the production of X-rays by electron guns. A considerable expansion and diversification of radiation hazards to medical technologists have resulted from the increasing use of radioistopes for diagnostic and therapeutic use into medical practice during the last 15 years. Unless such materials are handled with distinct caution, cancer hazards from contact with radioisotopes may ensue not

only for the regular technologists in charge of such materials but also for other members of the laboratory staff such as histological assistants, cleaning personnel and attendants of autopsy rooms.

The type of target organ of any cancerous response to such radioactive materials depends on the kind of radioisotope as well as on the route or contact or introduction into the body. Cancers of the skin may be caused by external exposures to x-radiation and emitters of beta- and gamma rays. The inhalation of radioactive gases and dust may elicit cancers of the lungs and nasal sinuses. Penetrating radiation by X-rays or deposition of radioactive substances after inhalation or ingestion in the bone, may produce cancers of the bone and leukemia (radiostrontium$^{90}$, radiophosphorus). Storage of radioactive matter such as thorotrast, in organs of the reticuloendothelial system, such as the liver and the spleen, may be followed by the development of hemangio-endotheliomas of these organs.

The appearance of cancers of the skin is usually preceded by the development of a chronic radiodermatitis, that of cancers of the bone by a chronic periostitis, and that of leukemia by aplastic (leukopenia, anemia), and hyperplastic (leucocytosis, monocytosis, leukemoid reactions) responses of the blood-forming tissues. The latent period may range from 2 to 30 years, and therefore, may be much longer than the exposure time which with powerful exposures may be very short, i.e., minutes. Radiation burns of the skin produced by a single exposure to X-rays may give rise to cancers of the skin appearing many years later.

In addition to these exposures to recognized occupational carcinogens, medical technologists may have contact with a number of potential human carcinogens, i.e., chemicals for which the available incriminating evidence concerning their carcinogenic properties for man is controversial, or for which only positive observations on experimental animals exist. Carcinogens of this type are natural and synthetic estrogens, carbon tetrachloride, chloroform, diethylene glycol, thiourea derivatives, and beta-propiolactone.

In summary it may be stated that medical technologists may become effectively exposed to a number of recognized or suspected human carcinogens during the performance of their regular work. Cancer hazards associated with such exposures may effectively be prevented by the observation of suitable precautionary measures. Technologists, therefore, should be aware of these hazards and should familiarize themselves with the proper control measures for preventing the development of such avoidable occupational cancers.

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# RADIATION AND CANCER†

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## Introduction

High-energy radiation (e.g., X or gamma rays, beta or alpha particles, or fast neutrons) have a special effect when they interact with material of any kind. This special effect arises from ionization, and the rays or particles that produce it are called ionizing radiations.[2,10] When a large amount of ionization from these sources occurs in living tissues, the cells making up the tissues are injured or destroyed, and cancer may ensue.

The series of events during the months or years between the original ionizations in the tissue and final development of a cancer are poorly understood. Some of the ideas on how ionizing radiation produces cancer are presented later. We know that, under some circumstances, ionizing radiation is carcinogenic to both experimental animals and man.

As might be expected, many variables must be considered in discussing radiation-induced cancer. These fall into the broad groups of radiation factors, i.e., the several variables that physically describe the ionizing radiation to which the tissues are exposed and the group of host or tissue factors. The latter factors are biological variables and, because of their apparently infinite number and complexity, limit our ability to describe them.

The interaction of these two sets of factors describe the field of radiation biology, of which radiation cancer is an especially important part.

## Radiation Factors

**Types of radiations.** Several different types of ionizing radiations make up two main classes—electromagnetic and corpuscular. X and gamma rays are in the first class; beta particles (electrons), alpha particles, and neutrons are the most commonly encountered corpuscular ionizing radiations. Some radioactive substances, such as radium, give off both electromagnetic and corpuscular radiations.

All the types mentioned can produce cancer in man or experimental animals. This fact, plus other effects of ionizing radiations, allows us to conclude that qualitative responses to the different types of radiation are the same since, in all cases, ionization is the primary physical event. This is not true of quantitative responses to the different types since the corpuscular radiations cause more damage per unit length of tissue they pass through than electromagnetic radiation of the same energy. Thus one of the most important parameters to be considered is the spatial distribution of ionization in an irradiated material.

As illustrations of these conclusions, we can cite the production of skin cancer by X rays, gamma rays, or beta particles.[4] Bone tumors can be produced by X or gamma rays, but it is much easier to produce them with beta or alpha particles. Leukemia can be induced by X rays, gamma rays, or neutrons from an external source or from an internal source such as parenterally injected radioactive phosphorus.[5]

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**Dose, intensity, and quality of radiation.** When a local area is exposed, usually a very high dose of radiation (thousands of roentgens) is required to produce a cancer; but a few hundren roentgens to the whole body may be carcinogenic. In either local or whole-body irradiation, a correlation is usually seen between the amount of radiation and the number of tumors produced. It is also generally true that the greater the radiation exposure the more quickly the cancer develops.

Many different units are used to describe the radiation dose, including the *roentgen*, the *rad*, *rem*, and *rep*.[11] The *rad*, a unit that gives the amount of energy absorbed per gram, is considered to be the most accurate way of describing the exposure dose.

Figure 1 illustrates the relation of X-ray dose to thymic leukemia incidence in mice; increasing the amount of radiation up to a certain point increases the number of cases of leukemia. Since thymic leukemia occurs in a certain percentage of nonirradiated control mice, it is not possible to decide whether there is a threshold amount of radiation necessary to produce a case of leukemia or any amount of radiation, no matter how small, increases an animal's chances of getting this form of cancer. The rate at which the exposure is given (radiation intensity) is also an important variable. It is usually thought that giving the total

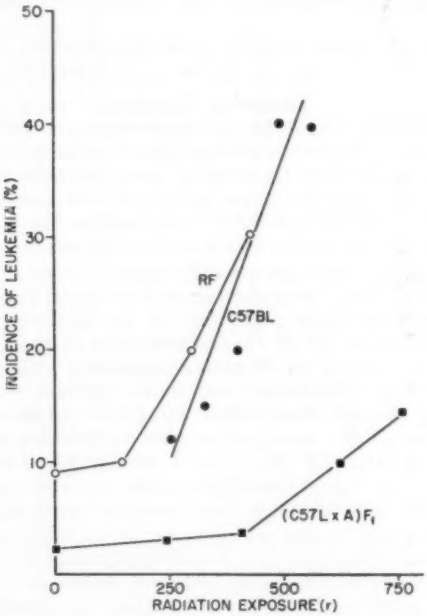


**Figure 1. Relation of radiation dose to incidence of leukemia in different strains of mice. Data from different sources, compiled by A. C. Upton, 1959.**

dose very slowly lessens the chance for tumor formation since a low intensity would allow some healing to occur. This is not true when the final total dose is extremely high.

Quality is another term frequently used to describe ionizing radiation. This applies to its ability to penetrate matter, as determined by the initial energy of the radiation; e.g., 1000-kv X rays are much more penetrating than 50-kv X rays. The effect of this variable on cancer production is probably overshadowed by the more-significant variables of dose and intensity.

## Host Factors

As mentioned earlier, the radiation factors can operate on innumerable biological variations. Site of irradiation—whether local, partial body, or whole body—is a major consideration in the development of a tumor. In addition, type of animal, age, diet, sex, endocrine status, presence or absence of certain organs, and other pathologic phenomena coexisting with the radiation exposure are some of the host factors that have been studied.[13,14] Probably more is known about these factors in the radiation induction of thymic leukemia in mice than for any other tumor situation. Figure 2 shows an example of the relation of age of mice at time

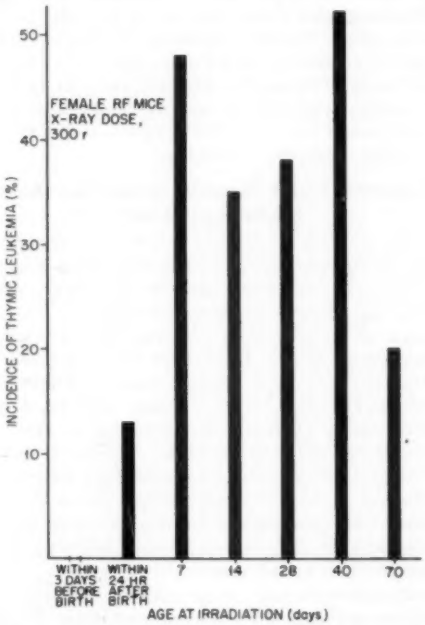


**Figure 2. Relation of age of mice at time of X irradiation to leukemia incidence. Data taken from A. C. Upton, E. P. Sniffen, and F. F. Wolff. Proc. Amer. Assoc. Cancer Res. 3: 71, 1959 and unpublished data.**

of irradiation to the production of leukemia.

## Common Sites of Radiation Cancers

It is frequently pointed out that, within 7 years after the discovery of X rays in 1895, it was known that skin damage caused by the X-ray ionization in that tissue could result in cancer.[12,16]

Natural radioactivity was discovered in 1896, but it was not until 1918 that Lazarus-Barlow showed that exposure to radium could produce skin cancer in mice.[4,8] A "radium cancer" occurring on the thumb of a man handling radium salts was first described in 1927.[15]

As the tragic historical lesson clearly shows, most radiation-induced cancers in man developed in the skin. The majority of these skin cancers were seen in the radiologists, who exposed themselves to X rays in the years when radiology was a new branch of medicine. Some occurred in persons who handled radium and its decay products before the carcinogenicity of emitted beta and gamma rays was realized.

It is now thought that any tissue containing cells capable of dividing can develop a neoplasm. Experience has shown, however, that some exposure situations and certain tissues are more likely than others to be involved in radiation cancer.

In addition to radiation-induced skin cancer, leukemia, bone tumors, and tumors of the lung and liver can occur in man as a result of ionizing irradiation. In experimental animals endocrine tumors, intestinal cancer, and a variety of other neoplasms can also be induced by radiation. From whole-body irradiation experiments in mice, it is clear that the incidence of many naturally occurring tumors in a given strain can be significantly increased but in some strains of mice the incidence of a particular tumor may actually decrease.

## Special Occupational and Medical Situations Associated with Radiation Cancer

Several situations of radiation cancer in man are now on record besides those involving the pioneers in radiology and others occupationally exposed during the years medical people were learning how to handle ionizing radiations for diagnosis and therapy.

A high incidence of lung cancer in uranium ore miners of Bohemia has been noted for many years. Radiation exposure in the miners is one of the possible causes of these cancer cases.[5] Osteogenic sarcoma in women who paint watch dials with radium and its decay products has been the subject of many studies.[5] In these cases the radioactive material was taken inadvertently by mouth. At one time radium chloride was given as a medical therapeutic measure to patients with a variety of medical complaints. Some of these people have developed malignant tumors. In the latter two groups, the over-all cancer incidence in exposed persons was estimated at 20 per cent with a latent period for appearance of the tumor of 19.5 to 31 years.[5]

Thorium dioxide (thorotrast) was widely used for some time as a contrast medium for making tissues radio-opaque. Eleven cases of cancer are now attributed to this agent, which emits alpha particles. Thorium dioxide is deposited in the liver; and several cases of liver cancer have been attributed to its alpha emission.[5]

Much interest now centers around the radioisotopes being used in medicine and industry and the radiation we receive from atomic bomb fallout. The amount of radiation received in medical and dental X-ray diagnosis is also being carefully studied.

Since our tissues and our natural environment contain radioactive materials, even in the absence of all the man-made sources, how much radiation it takes to produce a cancer has become an essential question.

## Mechanism of Radiation Cancer Production

One of the biologic effects of ionizing radiations is mutation in genetic material. Mutation is easily demonstrated by exposing germinal tissue cells of species for which suitable genetic markers are available. Mutation of cells in somatic tissues is much harder to demonstrate since genetic markers for such cells are not readily available for study. Cancer induction, whether by chemicals, viruses, or radiations, is often suggested to exemplify somatic mutation.

One theory is that ionizing radiations cause a somatic mutation in the cells of tissues they pass through, and the expression of the mutation in dividing cells may be unlimited and uncontrolled growth, or cancer.[3]

Another mechanism that is being suggested for radiation-induced leukemia in mice is that the destruction of tissues by whole-body irradiation releases a latent virus, which then acts on susceptible tissues such as the thymus to produce the leukemic process. The liberated virus could still act by a mutation process, but so little is known about the details of virus action on mutation production that the basic issue remains unsettled.[9]

A third mechanism of radiation cancer production is of considerable experimental interest. It applies to situations where irradiation of one organ leads to cancer in another organ. As might be expected, such interrelations can be produced in the endocrine system. If the thyroid gland is exposed to large doses of radioactive iodine and is destroyed, then its hormonal products are not available to regulate the growth of the pituiary gland. Pituitary tumors in mice can be produced in this way. If the mouse ovaries are damaged by radiation so that they cannot regulate the amount of gonadotrophin produced by the pituitary, then that organ will secrete large amounts of gonadotrophic hormone and cause a tumor of the ovarian cells not killed by the original irradiation. These indirect action mechanisms of radiation cancer production depend on either excessive hormonal stimulation of the target organ or a lack of hormonal control over it. Artificially restoring the endocrine balance prevents the formation of this type of radiation cancer.[3]

Kaplan has used these types of experiments as models for explaining all tumor formation.[7] The lack of thyroid hormone leads to an uncompensated growth of the pituitary, causing its continuous proliferation, which finally escapes all regulation to become a tumor. In the experiments on ovarian tumor production, the gonadotrophin from the pituitary continually stimulates certain of the ovarian cells until they become cancerous. Here, too, we could claim that the final result of the uncompensated growth stimulus was a somatic mutation.

## Prevention of Radiation Cancer

If people who handle radioactive materials or other radiation sources are adequately informed on the range of powerful biologic effects these materials may produce, human cancer resulting from careless use of these materials or sources is needless. Avoidance of exposure beyond the permissible levels is an adequate control measure, which would have prevented all the cases mentioned in which radiation was clearly established as a cause of the cancer. We would say now that our most effective means of preventing radiation cancer is education in the proper techniques for handling ionizing radiation.

A more difficult problem, and one that cannot be settled until more thorough experiments on radiation induction of cancer are undertaken, is how little radiation it takes to produce some increase in cancer incidence in a population. Extended discussions now center around the question of whether there is a threshold dose of radiation needed to initiate a cancer or whether even extremely small doses have a statistical chance of causing a tumor. Our final informed attitude toward "permissible" occupational exposures, medical diagnostic exposures, fallout, and natural environmental irradiation depends on the answer to these questions.

In the laboratory, prevention of radiation cancer may be approached by other means than avoidance of radiation. All these pertain to radiation situations in which a very high incidence of tumors is produced. Some chemical substances given to mice shortly before they are irradiated will reduce the magnitude of the radiation effect. The effect of these substances on radiation-induced cancer is being studied.

Radiation-induced thymic leukemia in mice can be prevented by giving a bone marrow or spleen injection to the animal after exposure.[6]

The radiation-induced tumors that depend on a hormonal imbalance for their genesis can be prevented by correcting the hormonal imbalance.[5]

Beyond these approaches, there are now basic studies in genetics suggesting that radiation-induced mutational processes can be interfered with or prevented if certain metabolic treatments are carried out shortly after exposure.[1] Viruses (if they are important in radiation cancer) are being intensively studied as cancer-producing agents in nonirradiated animals.

None of these experimental approaches have reached a point significant to clinical application, but they are vital to our understanding of radiation carcinogenesis.

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# HEMOGLOBIN ELECTROPHORESIS*

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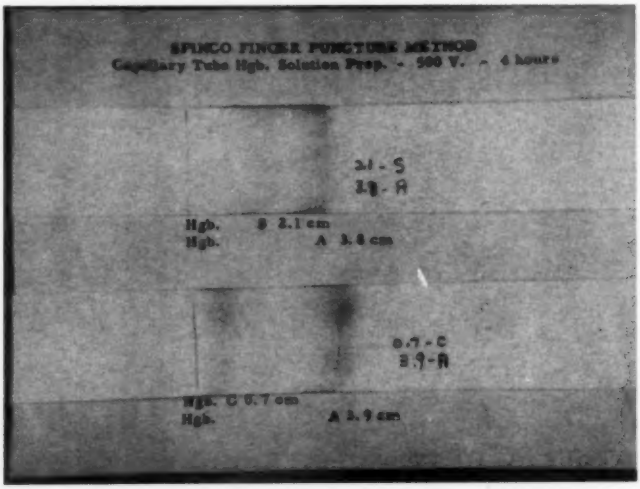
Because of unfamiliarity with existing methods, a rapid method for the preparation and electrophoretic separation of hemoglobin solutions is not available in many laboratories. For hemoglobin electrophoresis to become practical in the general laboratory, the procedure must be rapid in the preparation of a hemoglobin solution and must result in a wide migration pattern in the shortest time possible. Various early methods, unsuitable for routine use, have required about twenty hours total time for the preparation of the hemoglobin solution and for the electrophoretic separation of the molecules.

In literature, there are numerous methods described which would seem rapid enough to be of practical value. Many methods and various modifications were compared in an effort to find one most suitable for our use. Studies in this laboratory led us to choose the "Finger Puncture Technique" by Spinco[9] as a method of hemoglobin electrophoresis which is rapid enough to be a routine laboratory procedure.


SPINCO FINGER PUNCTURE METHOD
Capillary Tube Hgb. Solution Prep. - 500 V. - 4 hours
2.1 - S
2.9 - A
Hgb. S 2.1 cm
Hgb. A 2.9 cm
0.7 - C
2.9 - A
Hgb. C 0.7 cm
Hgb. A 2.9 cm

The following are methods, results, and observations from which our conclusion was drawn:

I Spinco Operating Instructions[7]

a) Hemoglobin solution prepared according to Drabkin[4]

b) Buffer: 1.84 grams diethyl barbituric acid
10.30 grams sodium diethyl barbiturate

* Received for publication February, 1960.

Water up to one liter
Ionic strength—0.05

c) Whatman 3 mm strips (30.6 x 3.0 cm.)
d) Dilute hemoglobin sample to 10% and apply 0.01 ml. in the form of a stripe
e) Separate at 22 ma per cell for four hours
f) Approximate migration: hemoglobin C 0.4 cm.
hemoglobin S 0.8 cm.
hemoglobin F 1.3 cm.
hemoglobin A 1.6 cm.
g) Oven drying temperature 110°-120° C
h) The Drabkin's preparation required about 1 - 2 hours. The resulting migratory pattern is so short, after four hours separation, accurate interpretation is unlikely.

II Spinco Technical Bulletin[8]

a) Hemoglobin solutions were prepared by a modified Drabkin's technique.
b) Buffer: 2.76 grams diethyl barbituric acid
15.40 grams sodium diethyl barbiturate
Water up to one liter
Ionic strength 0.075
250 cc. of this stock solution is diluted to one liter with a resulting ionic strength of 0.019.
c) Schleicher and Schuell 2043A strips 3.0 x 30.5 cm.
d) 0.01 ml. of prepared sample is applied in the form of a stripe.
e) Separate at 2.3 ma per cell for 16 hours.
f) Dry at 110°-120° C and stain with bromphenol blue: Scan.
g) Approximate migration: hemoglobin C 1.2-1.5 cm.
hemoglobin S 3.7-4.1 cm.
hemoglobin F 5.4 cm.
hemoglobin A 5.9-7.5 cm.
h) This is the most lengthy procedure attempted. The pattern must be stained and scanned on the Analytrol after a 16 hour separation. The migrated pattern is usually too faint for visual interpretation, depending on the patient's hemoglobin concentration. Reproducible results have not always been obtained by this lengthy procedure.

III Veterans Administration Hospital[11]

a) Hemoglobin solutions are prepared according to the original Drabkin[4] method and separated at once.
b) Buffer: 1.84 grams diethyl barbituric acid
10.30 grams sodium diethyl barbiturate with water up to one liter
pH-8.6. Ionic strength 0.05
c) Whatman 3 mm sheet—10 x 12.5 inches
d) Apply five lambda of prepared undiluted sample per spot. Nine or more samples may be placed on each sheet of paper.
e) Separate at 200 volts for fourteen hours

f) Approximate migration: hemoglobin C 0.8 cm.
hemoglobin S 1.5-2.0 cm.
hemoglobin F 2.1-2.6 cm.
hemoglobin A 2.8-3.1 cm.

g) Oven temperature 110°-120° C.

h) With this method there is not a clear separation of components due to both the relatively short length of the migration reached after fourteen hours of separation and the degree of freshness of the Drabkin's solution.

IV This procedure is identical to III, but, instead of one sheet of Whatman 3 mm. 10 x 12.5 inches, eight strips of 3.0 x 30.6 cm. were used.

a) Approximate migration: hemoglobin C 1.0 cm.
hemoglobin S 2.2-2.6 cm.
hemoglobin F 3.0-3.1 cm.
hemoglobin A 3.3-3.4 cm.

b) This method results in a slightly wider migration, but, as before, there was not a clear separation of components.

V This again is a variation of method number III with the sample applied in a stripe using about 0.01 ml. on strips of 3.0 x 30.6 cm. paper.

a) Whatman 3 mm. strips

b) Approximate migration: hemoglobin C 1.8 cm.
hemoglobin S 3.7-4.0 cm.
hemoglobin F 5.0 cm.
hemoglobin A 5.0 cm.

c) Schleicher and Schuell 2043A strip

d) Approximate migration: hemoglobin C 1.0 cm.
hemoglobin S 2.9 cm.
hemoglobin F 4.2 cm.
hemoglobin A 4.2 cm.

e) It was noted that this procedure, which employed the Whatman 3 mm. strips and the sample in the form of a stripe gives a much better length of migration and more clear separation. There remains, however, the problem of lengthy preparation of the Drabkin's solution and the fourteen hour separation of the hemoglobin molecules on the cell.

VI Spinco Hemoglobin Analysis: Finger Puncture technique

a) The hemoglobin sample is prepared in the following manner:

(1) Finger puncture is made

(2) In a capillary tube (1.2-1.4 mm. x 75 mm.), one centimeter length is filled with blood.

(3) About four centimeters of distilled water is drawn up into the tube.

(4) This mixture is allowed to hemolyse for at least fifteen minutes.

(5) Seal the end of the tube-commercial sealing putty is good.

(6) Centrifuge at high speed for fifteen minutes, (11,000 rpm in Micro. Hmt. cent.)

b) Buffer: 1.84 grams diethyl barbituric acid.
10.30 grams sodium diethyl barbiturate with water up to one liter.
0.05 Ionic strength
For the working buffer, take 250 ml. of the stock buffer and add 750 ml. of water.

c) Whatman 3 mm. strips 3.0 x 30.6 cm.

d) To apply the sample: break off the sealed portion of the capillary tube which contains packed ghost cells. Discard. Apply a volume of hemoglobin to each strip which occupies two to four centimeters of the tube. The hemoglobin is applied by gently drawing the tube back and forth across the paper strip.

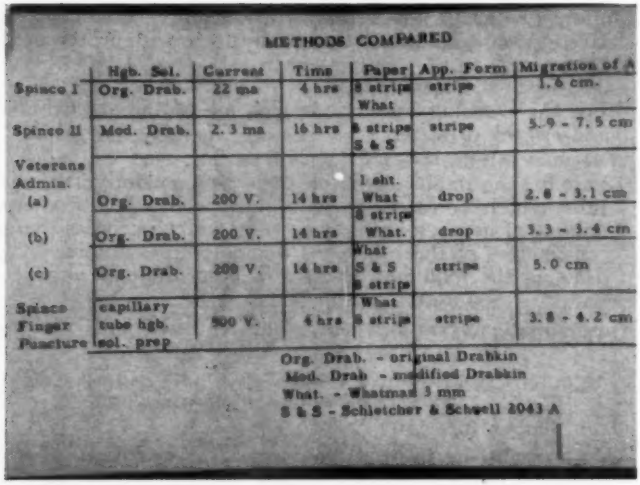
e) Separate at 500 volts for four hours.

f) Approximate migration: hemoglobin C 0.4-0.7 cm.
hemoglobin S 2.0-2.3 cm.
hemoglobin F ?
hemoglobin A 3.8-4.2 cm.

g) Oven temperature 110°-120° C.

h) This procedure is basically the same as it was received from the Spinco Company with a slight variation. Herewith is the method including our modifications.
Day old samples were prepared, separated as stated, and produced very little or no variation from the original pattern. The procedure employing an ammonium-potassium oxalate mixture and blood was also used with no apparent variation in results. As the Spinco directions state, the technique is "designed to be simple, economical, requiring minimum technician time to determine the presence of hemoglobin S in blood samples."[9] This method was found most suitable for our laboratory. The results were reproducible and the varied hemoglobin components were easily identified.

After repeated separations with each of the outlined techniques, it was concluded that for a rapid, simple separation, resulting in up to 4.5 cm. migration for hemoglobin A the "finger puncture technique," as outlined by Spinco, proved most suitable. Within one half hour from the time the blood is drawn, it can be on the cell and separating; whereas, with other techniques, up to two hours may be required before the solution is ready. Some of the adapted techniques for the preparation of Drabkin's hemoglobin solution are more time consuming because it is necessary to refrigerate overnight or freeze and thaw the sample to lyse the cells. In the finger puncture technique, the prepared hemoglobin solution is placed on the cell in one-half hour from the time it is drawn; and, in four hours, the separation is complete. Total time is four hours and forty-five minutes, including lysing, centrifuging, separation and drying. The separation of hemoglobin F is not fully obtained with this method, as is also true in other procedures. However, the band of hemoglobin AF is wider using the "finger puncture technique" than the band of hemoglobin A alone. When a wider band is encountered, the presence of hemoglobin F may be verified by performing the alkali denaturization technique for fetal hemoglobin.



METHODS COMPARED

| | Hgb. Sol. | Current | Time | Paper | App. Form | Migration of A |
|---|---|---|---|---|---|---|
| Spinco I | Org. Drab. | 22 ma | 4 hrs | 8 strips What | stripe | 1.6 cm. |
| Spinco II | Mod. Drab. | 2.3 ma | 16 hrs | 8 strips S & S | stripe | 5.9 - 7.5 cm |
| Veterans Admin. (a) | Org. Drab. | 200 V. | 14 hrs | 1 sht. What | drop | 2.8 - 3.1 cm |
| (b) | Org. Drab. | 200 V. | 14 hrs | 8 strips What. | drop | 3.3 - 3.4 cm |
| (c) | Org. Drab. | 200 V. | 14 hrs | What S & S 8 strips | stripe | 5.0 cm |
| Spinco Finger Puncture | capillary tube hgb. sol. prep | 500 V. | 4 hrs | What 8 strips | stripe | 3.8 - 4.2 cm |

Org. Drab. - original Drabkin
Mod. Drab - modified Drabkin
What. - Whatman 3 mm
S & S - Schleicher & Schuell 2043 A

## Summary

Various hemoglobin electrophoresis methods have been reviewed and the advantages and/or disadvantages of each have been discussed, the Spinco "finger puncture technique" with direct applications to the electrophoresis paper, has proved the most simple, rapid and economical procedure studied. It offers a clear separation of hemoglobin components C, S, A, which are the ones most frequently encountered. The hemoglobin A migrates fairly constantly to 4.3 cm and the total time required is only four and three-quarter hours.

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# A STUDY ON AN ULTRAMICRO AND AUTOMATED PROCEDURE FOR SERUM PROTEINS*

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The estimation of the concentration of serum albumin in minute volumes of material using salt fractionation is time-consuming and somewhat difficult. We have therefore adapted a rapid and technically simple procedure for the ultramicro determination of serum albumin.[1] When this method is coupled with a biuret technic for total serum protein,[4,7] the determination of serum albumin and globulin is greatly simplified and applicable to the pediatric circumstance.

A selective reaction, such as albumin absorption of an anionic dye with a subsequent shift in its absorption spectrum, appeared most suitable. Previous studies have indicated that serum albumin, in contrast to other serum fractions, is unique in its affinity for certain anionic dyes.[3,5,8] Because of this affinity, Brachen and Klotz[1] proposed a simple and rapid method for the determination of serum albumin, utilizing a buffered methyl orange reagent. The methyl orange absorption procedure was selected for the present investigation because it has been studied extensively,[1,3] and because it has been applied successfully to the automatic determination of serum proteins.[2] However, several important facets of this technic were not discussed and these include the following points:

(1) In attempting to change from an automatic to a manual microprocedure for serum albumin, it was found that there was a disturbing effect on the manual calibration curve when the ambient temperature varied. This appeared to cause differences in the observed data with known concentrations of albumin over the temperature range encountered in everyday determinations. Therefore, the effect of change in temperature was studied quite carefully for the albumin-methyl orange reaction for this ultramicro application.

(2) The effect of increasing the ratio of methyl orange reagent to serum was also investigated since the concentration range covered by the calibration curve using the original technic was not as steep as it might be in the 4-6 gm. per 100 ml. range and yet was very steep in the lower ranges of concentration.

(3) The effect of the natural pathological variable bilirubin was studied. Its influence on the methyl orange reaction has not been determined. Nor has any possible detrimental effect on the biuret reaction which is used for the total protein reaction been determined.

(4) The differential absorbance characteristics of the methyl orange and methyl orange-albumin system were studied to determine the wavelength of maximum differential between the dye and the dye-albumin complex.

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(5) A modification in the manifold of the original automation procedure for albumin was made which takes advantage of the steeper portion of the calibration curve. Also, the principle of scale expansion was employed for the albumin-methyl orange system which increased the width of the recordings obtained and assured greater sensitivity.

## REAGENTS AND PROCEDURE

**Buffered methyl orange, pH 3.5.** Dissolve 21 gm. of citric acid in 1 liter of 0.2 N sodium hydroxide. Adjust the pH to exactly 3.5 with concentrated hydrochloric acid. Dilute 25 ml. of 0.1 per cent aqueous methyl orange solution to a liter with the buffer. This solution should have an absorbance value at 5-15°C between 0.85 and 0.90 when read against a distilled water blank at 535 mu employing a 1 cm. light path microcuvet in a Beckman DU spectrophotometer. If the value does not fall within these limits, simply adjust the absorbance with 0.1 per cent methyl orange or the citrate buffer. This reagent is stabilized if a few drops of liquid cresol are added to prevent the growth of fungus.[1]

**Stock biuret reagent.** Dissolve 45 gm. of sodium potassium tartrate in about 200 ml. of 0.2 N sodium hydroxide. Add, dissolving separately, 5.0 gm. of copper sulfate ($CuS0_4 \cdot 5H_2O$) and 5.0 gm. of potassium iodide, dilute the solution to a liter with 0.2 N sodium hydroxide, and mix well.[7] Dilute the stock biuret reagent 1:1 with distilled water to prepare a working reagent for the manual method.

**Stock standard protein solution.** Determine the protein concentration of 25 per cent normal human serum albumin (salt-poor) either by biuret or Kjeldahl analysis. Dilute this albumin to 10 gm. per 100 ml. with sodium chloride solution so that the final concentration of salt is 0.85 per cent. The solution is stable for at least one month if refrigerated.

**Diluted standard protein solutions.** Pipet 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 ml. of the stock standard protein solution into 10 ml. volumetric flasks and dilute the contents of the flasks to the mark with 0.85 per cent sodium chloride solution, mix well, and refrigerate.

**Standardization and procedure, serum albumin.** Pipet 1.0 ml. of the methyl orange reagent into each of eight 70 by 7 mm. test tubes and place the tubes in an ice bath. However, any preferred temperature extending from that of the ice bath to room temperature can be used. Into one tube, pipet 25 lambdas of 0.85 per cent sodium chloride solution. Into each of the other tubes pipet 25 lambdas of the diluted protein standard solutions which range in concentration from 2.0-6.0 gm. per 100 ml. Mix by inversion. Within one-half hour determine the absorbance of the solutions at 535 mu using a microcuvet in a Beckman DU spectrophotometer. Distilled water is used to represent 0.0 absorbance. Any other sensitive spectrophotometer which can be adapted to received microcuvets should be satisfactory. From these data prepare a concentration-absorbance curve. Determine the albumin concentration of the unknowns by substituting 25 lambdas of serum for the standards and treating them as described.

**Standardization and procedure, total serum protein.** Pipet 1.0 ml. of diluted biuret reagent into each of nine 70 by 7 mm. test tubes. Into one tube pipet 25 lambdas of 0.85 per cent sodium chloride solution.

Into the other tubes pipet 25 lambdas of the diluted protein standard solutions and mix the solutions by inversion. After thirty minutes determine the absorbance of each at 535 mu against the saline blank solution. From these data prepare a standard concentration-absorbance curve. Determine the protein content of the unknowns by substituting 25 lambdas of serum for the standards and treating them as described.

**Procedure, automation.** The photometer-recorder system is zeroed at 536 mu, (1 cm. light path) with water in the sample line diluting the biuret solution coming in through two reagent lines. The standards, encompassing the range of 3.0-8.0 g. per 100 ml. and the unknowns are sampled then at the rate of 40 per hour. The same manifold, filters and flow cuvet are used for serum albumin. Zeroing is accomplished with water pumping through all lines and then the methyl orange reagent is aspirated through the reagent lines to record the base line. The 100% T control is then increased by 300 thus shifting the base line to the right. The standards 2.0-6.0 g. per 100 ml. and the unknowns are then sampled using this scale expansion system at the rate of 40 per hour.

## DISCUSSION AND RESULTS

Figure 1 shows a series of calibration curves graphed from the data obtained with the same albumin-methyl orange solution where the only variable was temperature. It can be seen that the blank increases in absorbance with a decrease in temperature whereas just the opposite effect is encountered when one adds albumin to the dye reagent. A cross-over point of equal absorbance is the isosbestic point achieved at 1 gm. per 100 ml. of albumin. There is very little difference between the 5°C.

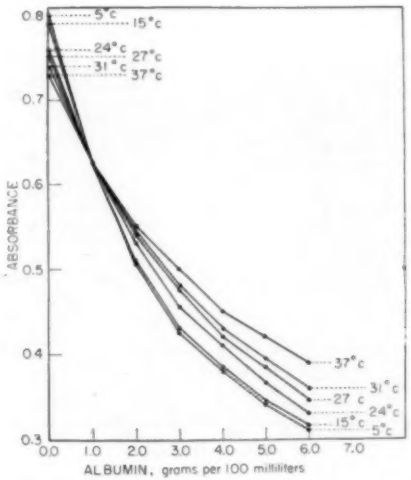


**Figure 1. Effect of temperature on the albumin-methyl orange reaction.**

and 15°C. calibration curves. Therefore, if one cools the solutions in an ice bath prior to measurement, it is quite simple to measure the absorbance at an in between temperature with no loss in accuracy through the use of a calibration curve. Measurements made on different days yielded a reproducible collection of data with an excellent degree of precision. Any controlled temperature as shown in the graph may be used. The ratio of serum to methyl orange in this experiment was 1:20 as described by the original authors. An increase in the ratio of reagent to serum as described under procedure decreases this effect of temperature.

Since bilirubin is found in the albumin fraction of serum, its effect on the albumin analysis was investigated for both the biuret and the methyl orange reactions. Varying concentrations of bilirubin covering the range of 0-40 mg. per 100 ml. was added to 4 g. per 100 ml. human albumin solution and the biuret reaction[7] was carried out. The colors of the several solutions varied from purple in the bilirubin uncontaminated solution to blue in the 40 mg. per 100 ml. solution. The error for the 40 mg. per 100 ml. sample calculated at 18 per cent. The effect of bilirubin on the methyl orange reaction showed an error well below 5 per cent when 20 mg. per 100 ml. bilirubin was added to albumin and did not appear to contribute any significant interference.

Figure 2, curve B, describes a graphing of the spectrum of methyl orange at pH 3.5. When an identical solution was treated with a small volume of a concentrated solution of human albumin, a hypochromic shift in the spectrum of the dye was obtained and this is shown as curve A. Since the absorbance difference between the two solutions is the calibration device, a difference spectrum was obtained by the simple expedient of graphing one dye form versus the other dye form using

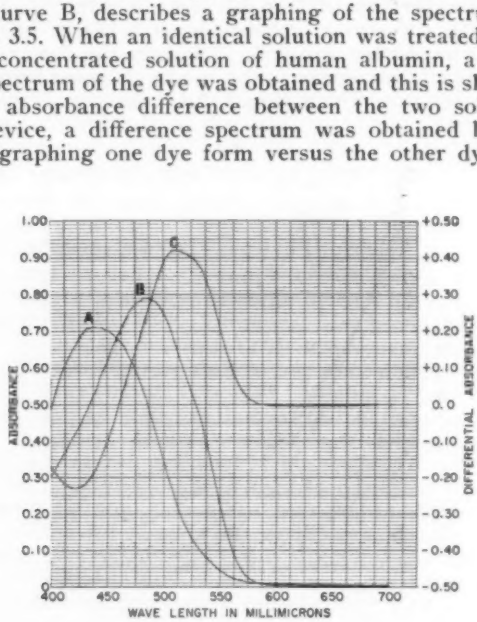


**Figure 2. Curve B, normal visible spectra for methyl orange reagent, Curve A, albumin-methyl orange complex and Curve C, difference spectrum for the subtractive effect.**

the differential setting of an automatic recording spectrophotometer. It can be seen that the greatest positive absorbance difference was obtained at 510 millimicrons and the greatest negative absorbance difference at 418 mu. Very little loss in sensitivity is encountered at 535 mu where there is less chance of interference from the spectrum of bilirubin. Although 550 mu is recommended by the original authors and this wavelength can be used, it represents a much smaller percentage of the peak absorbance than does 535 mu when using the Beckman spectrophotometer and is less sensitive. The wavelength of negative differential absorbance would be severely interfered with by bilirubin and it was therefore not considered for the determination.

Figure 3 shows the automation schematic including the simple manifold of the system used for both albumin and total protein. Although either the 6 mm. or 10 mm. cuvet can be used, the 10 mm. cuvet with a 1.67 factor of increase of sensitivity is preferable. Larger reagent tubings are used than previously described,[2] and these allow complex formation of albumin and methyl orange to take place in the steeper portion of the calibration curve. The reaction for total protein uses one coil at room temperature and a second coil in a 37°C water bath which ensures completeness of reaction. The two coils could be in a single water bath at 37°C, but they were used as shown, where the room temperature coil is the unplugged regular heating bath. The extra coil at 37°C is adequate for complete color formation. Both coils are bypassed for the albumin determination because the reaction between albumin and methyl orange is instantaneous. Beyond good mixing, an increase in reaction time is unnecessary.

The principle of scale expansion is used for the methyl orange reaction. Its function is to stretch the entire curve over a wide portion of the recording. This is accomplished by zeroing the instrument in the normal fashion at the prescribed wavelength with the reagent pump-

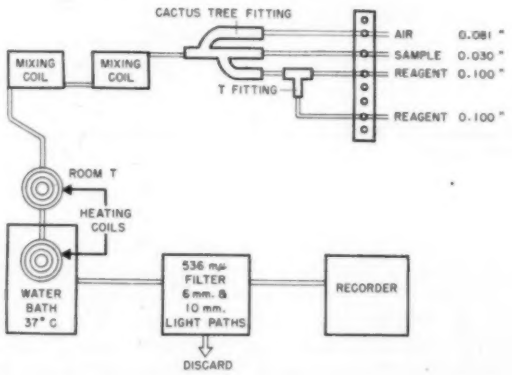


**Figure 3. Schematic diagram of the automation system for the serum proteins.**

ing through the photometer. Then, by the simple expedient of increasing the zero potentiometer reading, the curve is elongated and covers a larger percentage of the recorder chart. This effect allows an apparent increase in steepness with no loss in accuracy. Actually, the accuracy of reading is increased since the scale of the calibration curve is expanded.[6]

Figure 4 shows a few recoveries for both total protein and albumin using the improved system. The manual results present are analytically similar to the automatic results found. The calibration curve shown for albumin is markedly increased in steepness over the one previously described[2] even though it represents a greater dilution of the serum. The total protein sensitivity is increased by the factor of cuvet length while the albumin sensitivity is increased by the described system of scale expansion.

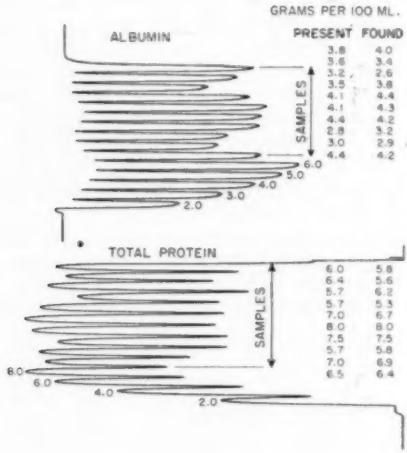


**Figure 4. Records obtained for calibration curves for the protein reactions and several recoveries with each system.**

Table 1 indicates the precision obtained for the two ultramicro manual determinations. Four consecutive analyses were performed on each of five serums for the determination of both albumin and total protein concentrations, to achieve the tabulated results shown.

Table 2 shows the acceptable agreement of values obtained by the use of the ultramicro methyl orange absorption technic with the values obtained by a standard biuret salt fraction method.[4,7] Total protein showed an equally favorable comparison to a standard biuret technic.[8]

**TABLE I**

**Precision of Ultramicro Methods for Serum Proteins**

| Serum Specimen | Albumin Gm. per 100 ml. Serum | Total Protein Gm. per 100 ml. Serum |
|---|---|---|
| 1 | 3.1 | 7.7 |
| | 3.1 | 7.7 |
| | 3.0 | 7.8 |
| | 3.0 | 7.6 |
| 2 | 3.3 | 6.6 |
| | 3.5 | 6.6 |
| | 3.3 | 6.5 |
| | 3.4 | 6.6 |
| 3 | 4.8 | 7.9 |
| | 4.7 | 7.9 |
| | 4.8 | 7.7 |
| | 4.8 | 7.8 |
| 4 | 2.4 | 7.0 |
| | 2.3 | 6.7 |
| | 2.4 | 6.8 |
| | 2.3 | 6.9 |
| 5 | 4.1 | 7.7 |
| | 4.2 | 7.8 |
| | 4.3 | 7.7 |
| | 4.3 | 7.6 |

**TABLE II**

**Comparison of Values Obtained by Standard and Ultramicro Procedures**

| Albumin Gm. per 100 ml. Serum | | Total Protein Gm. per 100 ml. Serum | |
|---|---|---|---|
| Standard Method | Ultramicro Method | Standard Method | Ultramicro Method |
| 3.6 | 3.7 | 5.2 | 5.1 |
| 4.4 | 4.7 | 5.6 | 5.6 |
| 3.1 | 3.3 | 6.0 | 6.2 |
| 2.9 | 3.0 | 7.2 | 7.5 |
| 3.3 | 3.3 | 6.1 | 6.0 |
| 4.9 | 5.2 | 6.1 | 6.2 |
| 4.8 | 5.2 | 6.7 | 6.8 |
| 3.8 | 4.2 | 6.8 | 7.0 |
| 4.4 | 4.8 | 6.4 | 6.8 |
| 3.2 | 3.4 | 6.0 | 6.0 |
| 4.2 | 4.5 | 7.4 | 7.6 |
| 3.3 | 3.6 | 6.9 | 7.0 |
| 2.9 | 3.2 | 7.7 | 7.7 |
| 5.1 | 5.2 | 5.8 | 5.8 |
| 3.8 | 3.9 | 6.9 | 7.3 |

The results of a recovery experiment are tabulated in Table 3. Different amounts of protein were added to saline diluted serums of known concentrations and determinations were then made for total protein in one instance and for albumin in the other. The amounts of material found were in good accord with the calculated concentrations.

**TABLE III**

**Values for Serum Determined by Ultramicro Technics, Before and After Addition of Known Quantities**

| Serum Component Tested | Gm. per 100 ml. | | | |
|---|---|---|---|---|
| | Amount Originally Present | Amount Added | Calculated Amount | Amount Found |
| Albumin | 2.1 | 1.7 | 3.8 | 3.9 |
| | .. | 2.4 | 4.5 | 4.6 |
| | 2.4 | 1.6 | 4.0 | 3.9 |
| | .. | 2.1 | 4.5 | 4.6 |
| | 2.0 | 1.8 | 3.8 | 3.6 |
| | .. | 2.3 | 4.3 | 4.2 |
| | 1.9 | 1.9 | 3.8 | 3.9 |
| | .. | 2.3 | 4.2 | 4.4 |
| | 2.0 | 1.8 | 3.8 | 3.9 |
| | .. | 2.3 | 4.3 | 4.4 |
| Total Protein | 3.9 | 1.6 | 5.5 | 5.4 |
| | .. | 3.4 | 7.3 | 7.2 |
| | 5.1 | 0.8 | 5.9 | 5.9 |
| | .. | 2.7 | 7.8 | 7.8 |
| | 3.6 | 1.5 | 5.1 | 5.1 |
| | .. | 3.5 | 7.1 | 7.0 |
| | 4.3 | 1.2 | 5.5 | 5.7 |
| | .. | 3.2 | 7.5 | 7.5 |
| | 4.6 | 1.0 | 5.6 | 5.7 |
| | .. | 3.0 | 7.6 | 7.6 |

## SUMMARY

A rapid, simple ultramicro technic has been described for the determination of serum albumin which, when combined with a biuret technic for total serum protein, greatly simplified the ultramicro determination of the albumin-globulin ratio. Precision, comparison with a standard method and recovery of additions to known values, indicate that the ultramicro method is of routine clinical value. The albumin-methyl orange reaction was shown to be temperature dependent and a method to correct for this phenomenon was described. The effect of bilirubin on both the biuret and methyl orange reactions, and the effect of dilution on the methyl orange reaction was discussed. A modified scheme for automated analysis including scale expansion for albumin was shown.

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# A MODIFICATION OF THE NEUTRAL RED CYTOCHEMICAL TEST*

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## Introduction

For years research workers have sought a simple and rapid *in vitro* test for the differentiation and virulence testing of mycobacteria. Many such cytochemical tests have been described but, because of the inconsistencies in results, many of these tests have been disregarded. One procedure, however, the neutral red test of Dubos and Middlebrook,[1] has had rather widespread acceptance. In the original test bacilli were suspended in an alkaline buffer to which the dye neutral red was added. In alkaline solution this dye is yellow, however, should it become fixed by the cells, the latter acquire a red color. Dubos and Middlebrook noted that only virulent tubercle bacilli fixed the dye, whereas avirulent strains acquired no color. Dubos and Suter showed[2] that certain surface active agents and cationic substances interfered with the test. Notably, cultures grown in the presence of ammonium salts and asparagine (both common constituents in many tuberculosis media) failed to bind neutral red unless thoroughly washed prior to the test. For this reason, best results were noted when cells first were treated by two successive washings of one hour each in 50 per cent methanol.

In an attempt to overcome the time-consuming methanol washing procedure without sacrificing reliability, this laboratory has undertaken to modify the original neutral red test. The purpose of this study was to compare the Dubos-Middlebrook neutral red test with the Communicable Disease Center (CDC) modification from the standpoint of 1) ease of performance, 2) speed and 3) reliability.

## Materials and Methods

Preliminary comparison of the Dubos-Middlebrook neutral red test with the CDC modification of same was carried out using stock cultures of acid-fast bacilli whose pathogenicity for laboratory animals was well documented. Results of this initial study were so gratifying that it was decided to correlate animal virulence and neutral red reactions of reference cultures submitted to this laboratory for identification. This report, therefore, deals with 201 reference cultures of acid-fast bacilli submitted to this laboratory for typing. Animal virulence of these cultures was determined by inoculating two guinea pigs subcutaneously in the right groin; one guinea pig received 5 mg and the second 0.1 mg wet weight of organisms, using the Hopkins vaccine tube method of standardization.[5] All animals were sacrificed after six weeks of infection and the extent of disease recorded by the method used in this laboratory.[5]

Lowenstein-Jensen coagulated egg medium, which contains both asparagine and added mineral salts, was used in performance of the Dubos-Middlebrook neutral red test which requires the two step washing procedure. Duplicate cultures, grown on Herrold's egg yolk agar medium,[3] were used in the CDC modified neutral red test. Herrold's medium is notably different from Lowenstein-Jensen in its lack of asparagine and mineral salts. If the work of Dubos and Suter[2] is correct then we might

* Received for publication Oct. 1960.

assume that cultures grown on Herrold's medium would require little or no washing before testing.

The Dubos-Middlebrook test was performed as follows: 1) several colonies from Lowenstein medium were suspended in 5 ml. of 50% methanol, 2) the suspension was incubated at 37°C for one hour, 3) the methanol was decanted, 4) methanol washing and decanting was repeated, 5) to washed cells was added 5 ml. of buffer* and 1 ml. neutral red,** 6) this suspension was then mixed by gentle shaking and allowed to stand at room temperature for 30 minutes before results were read. In reading results, a sediment of cells having varying degrees of redness was called positive, whereas cells which acquired no color or appeared yellow were called negative.

The CDC modified test was performed by suspending several colonies grown on Herrold's egg yolk agar in 5 ml of the above buffer. One ml of neutral red solution was added to this bacterial suspension and the tubes were mixed by gentle shaking. After standing for 15 minutes at room temperature, results were read as in the Dubos-Middlebrook test.

In order to determine the need for the methanol washing procedure, the CDC test was duplicated using colonies grown on Lowenstein medium; because of its content of asparagine and cationic substances, colonies from Lowenstein medium presumably should be washed before testing. This method is hereinafter referred to as "wash control."

## Results

Data from this study are recorded in Table 1. Two hundred and one cultures were compared in this animal—cytochemical test study. Of the

**TABLE 1**

**Comparison of Results of Several Neutral Red Tests on Cultures of Known Virulence for Guinea Pigs**

| | NEUTRAL RED TESTS | | | | | |
|---|---|---|---|---|---|---|
| | CDC Modified (Herrold's Medium) | | "Wash Control" (CDC Test On L-J Media*) | | Dubos Middlebrook | |
| Animal Data | + | — | + | — | + | — |
| Guinea Pig Positive (100 cultures) | 96 | 2 | 28 | 16 | 80 | 20 |
| Guinea Pig Negative (101 cultures) | 17 | 84 | 25 | 34 | 53 | 48 |

* Because of the extremely erratic results with this "control", this particular test was discontinued after being performed on only 103 cultures.

100 cultures which produced progressive disease in guinea pigs, 96 per cent yielded positive results using the CDC modified neutral red test with cultures grown on Herrold's medium; only 80% were positive using the original Dubos-Middlebrook test. Careful scrutiny of Table 1 shows what appears to be a typographical error. Of the 100 guinea pig positive cultures, only 98 were examined by the CDC modified neutral red test. The reason for this apparent discrepancy is the fact that two of the guinea pig positive cultures failed to grow on Herrold's medium and hence were not tested.

* 5% NaCl plus 1% sodium barbitol
** 20 mg% aqueous neutral red

Of the 101 cultures which produced no disease in guinea pigs, 84 per cent were negative using the CDC test and 48 per cent negative by the Dubos-Middlebrook test. Speculations as to the reasons for these extremely variable results will be discussed.

As noted in Table 1, the "wash control" procedure was discontinued after 103 tests because of its extremely erratic results when compared with guinea pig virulence.

## Discussion

The criteria for the determination of type and virulence of mycobacteria have always been animal pathogenicity tests. Since some laboratories are ill-equipped and animals are not available for testing, simple but reliable cytochemical tests in lieu of animal inoculation may be able to provide some degree of typing and virulence determination. In this regard, there seems to be some degree of correlation between guinea pig pathogenicity and neutral red reaction.

The neutral red test of Dubos and Middlebrook is used by many laboratories as an aid in virulence determination. Because of the methanol washes needed to remove the masking effect of asparagine and other substances, the time consumed in performance of the original test is at least three hours. An attempt was made to negate the necessity for the methanol washing by growing the cultures on a medium containing a minimum of inhibitory compounds. In this study Herrold's egg yolk agar (notably different in its lack of asparagine and inorganic salts), was used. Duplicate cultures were grown on Lowenstein-Jensen and Herrold's egg yolk agar medium. Tests were performed in parallel so that there was no difference in the age of the culture nor in the buffered saline—neutral red solution. To determine the necessity of the methanol wash, a control test was run with colonies taken from Lowenstein medium and suspended in the buffer-neutral red solution without first washing in methanol; with the exception of the difference in medium this duplicated the CDC modification. As previously pointed out, the results of the test performed in this manner showed such poor correlation with guinea pig pathogenicity that it was discontinued after 103 cultures were investigated. Results of the Dubos-Middlebrook technique using methanol washed cells indicate that washing does favorably affect the test results.

Examination of the data reveal a good correlation between guinea pig positive cultures and neutral red test results. A comparison of neutral red results with guinea pig negative cultures, however, shows a much poorer correlation. For instance, in this study of 101 cultures which produced no disease whatever in guinea pigs, 17 were neutral red positive. Although this appears to be a rather poor correlation, an examination of these 17 cultures revealed that two were isoniazid resistant human strains and 15 were chromogenic. The presence of pigment in the chromogenic cultures may be confusing for two reasons: 1) the pigment itself may interfere with proper interpretation of the final color reading and 2) the pigmented cultures well may belong to one of the groups of unclassified acid-fast bacilli. It is a well known fact that while both unclassified acid-fast bacilli and isoniazid resistant strains of *M. tuberculosis*

frequently are incapable of infecting guinea pigs, both of these groups may infect man and, for that matter, might give a positive rather than a negative neutral red test. That this latter is true is borne out by work of Wayne et al.[7]

Although Herrold's egg yolk agar is not recommended for primary isolation of acid-fast bacilli, it is used by some laboratories precisely for that purpose. This laboratory would recommend, however, that Herrold's medium always be used in conjunction with some other good primary isolation medium, such as Lowenstein-Jensen. In this study two of the cultures failed to grow on Herrold's medium and no attempt was made to secure growth by subculturing from the Lowenstein slant. In order to have an available culture for neutral red testing, a tube of Herrold's medium may be incorporated in the media used for culture of diagnostic specimens, or a subculture may be made from all positives. This involves very little time in comparison to the time saved in actual performance of the test by being able to omit the two methanol washings.

Attempts are being made to achieve better correlation between animal pathogenicity data and the neutral red cytochemical test. Huppert et al[4] in the microcolonial neutral red tests makes some differentiation of atypicals based on the neutral red reaction and cording. Further studies by Wayne[8] describe semi-quantitative determinations of positiveness of cultures based on pH and time required for fading of bound dye from red to yellow. Wayne described some group characteristics observed by this method.

Earlier it was inferred that animal pathogenicity tests can no longer be relied upon for final typing and virulence testing of the mycobacteria. This fact has been made quite clear in recent years with our increased knowledge of the unclassified acid-fast bacilli.[6] Attempts are constantly being made to determine type and/or virulence of these anonymous strains using cytochemical procedures. One rather reliable procedure is the observation of cord formation and neutral red staining on a microcolonial scale using the techniques of Wayne et al.[4,7,8] However, since more than 90 per cent of the primary isolates from tuberculosis patients are human type tubercle bacilli and since the unclassified strains, which comprise only about one per cent of primary isolation, can be identified by other characteristics, the few false positive neutral red reactions obtained in the method herein described actually sacrifice little in the way of accuracy.

## Summary

A modification of the original neutral red test of Dubos and Middlebrook is presented. This alternate procedure provides for a considerable saving in test performance time and from all outward appearances apparently is more reliable than the original procedure.

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